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PROTEINS AND THE THEORY OF COLLOIDAL BEHAVIOR

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PROTEINS
AND THE THEORY OF COLLOIDAL BEHAVIOR

BY

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PREFACE

Colloid chemistry has been developed on the assumption that the ultimate unit in colloidal solutions is not the isolated molecule or ion but an aggregate of molecules or ions, the so-called micella of Naegeli. Since it seemed improbable that such aggregates could combine in stoichiometrical proportions with acids, alkalies, or salts, the conclusion was drawn that electrolytes were adsorbed on the surface of colloidal particles according to a purely empirical formula, Freundlich's adsorption formula.

The writer's investigations have led to the result that this last conclusion is based on a methodical error, as far as the proteins are concerned; namely, to the failure to measure the hydrogen ion concentration of the protein solutions, which happens to be one of the main variables. When the hydrogen ion concentrations are duly measured and considered, it is found that proteins combine with acids and alkalies according to the stoichiometrical laws of classical chemistry and that the chemistry of proteins does not differ from the chemistry of crystalloids.

As long as chemists continue to believe in the existence of a special colloid chemistry differing from the chemistry of crystalloids, it will remain impossible to explain the physical behavior of colloids in general and of proteins in particular. This state of affairs is reflected in the concluding remarks of Burton's interesting book on "The Physical Properties of Colloidal Solutions" published in 1920,

"We may very well conclude with the words used by the pioneer worker Zsigmondy, in closing his first account of the early work on colloidal solutions:

'From the foregoing outline no general theory of colloids can be given, for the study of colloids has become a great and extensive science, in the development of which many must assist; only when the voluminous material supplied by much physico-chemical research has been properly systematized, will the theory of colloidal solutions be raised from mere consideration of the similarities in special cases to the standing of an exact science.'"

Professor F. G. Donnan, of the University of London, announced in 1910 an ingenious theory of equilibria which are established when two solutions of electrolytes are separated by a membrane which is permeable to all except one ion. This theory was successfully applied by Procter and Wilson to the explanation of the influence of electrolytes on the swelling of gelatin. It will be shown in this volume that Donnan's theory of membrane equilibria furnishes a quantitative and mathematical explanation not only of swelling but of the colloidal behavior of protein solutions in general; namely, electrical charges, osmotic pressure, viscosity, and stability of suspensions. Such an application of Donnan's theory would have been impossible without the stoichiometrical proof that proteins form true ionizable salts with acids and alkalies. What was at first believed to be a new type of chemistry, namely colloid chemistry, with laws different from those of general chemistry, now seems to have been only an unrecognized equilibrium condition of classical chemistry; at least as far as the proteins are concerned. This does not detract from the importance of colloidal behavior for physiological and technical problems, but it completely changes the theoretical treatment of the subject.

Any rival theory which is intended to replace the Donnan theory must be able to accomplish at least as much as the Donnan theory, *i.e.*, it must give a quantitative, mathematical, and rationalistic explanation of the curves expressing the influence of hydrogen ion concentration, valency of ions, and concentration of electrolytes on colloidal behavior; and it must explain these curves not for one property alone but for all the properties, electrical charges, osmotic pressure, swelling, viscosity, and stability of solution, since all these properties are affected by electrolytes in a similar way.

The contents of the book are divided into two parts, one furnishing the proof of the stoichiometrical character of the reactions of proteins, the second developing a mathematical and quantitative theory of colloidal behavior on the basis of Donnan's theory of membrane equilibria.

The theory of colloidal behavior, as outlined in this book, can only be considered as a first approximation. Finer methods of experimentation will have to be introduced, many minor dis-

crepancies will have to be accounted for, and many additions made. It was, however, thought advisable to publish the book for the reason that the experimental facts are accumulating so rapidly that it is difficult for anyone to gather the leading ideas unless they are presented more systematically and with less detail than in the original publications. It was also thought advisable to avoid in this volume a discussion of the possible applications of the new theory to physiological and technical problems.

The writer wishes to express his appreciation to his technical assistants, Mr. M. Kunitz, and Mr. N. Wuest, for the skill and care shown in the measurements required for the experimental part of the work.

The writer's thanks are also due to Dr. John H. Northrop, Dr. D. I. Hitchcock, and Dr. Anne Leonard Loeb, who have read part or all of the manuscript and offered valuable suggestions; and to Dr. J. A. Wilson, who kindly read and revised the first part of the chapter on swelling and suggested to the writer the mathematical proof on page 143 of the book.

The writer is indebted to Miss N. Kobelt for the reading of the proof and for the index.

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PROTEINS
AND
THE THEORY OF COLLOIDAL BEHAVIOR

CHAPTER I

HISTORICAL INTRODUCTION

1. The Alleged Difference Between the Chemistry of Colloids and of Crystalloids

The distinction between crystalloids and colloids was proposed by Graham in 1861, the crystalloids being characterized by a tendency to form crystals when separating from a watery solution, and the colloids by a tendency to separate out in the form of "gelatinous" (or amorphous) masses. Graham found that these two groups of substances differ also in two other respects, first, in their "diffusive mobility," and second, in a peculiar "physical aggregation." The crystalloids diffuse readily through different kinds of membranes (*e.g.*, pig's bladder, parchment) through which colloids can diffuse not at all or only very slowly. The second peculiarity is the tendency of the colloids to form aggregates when in solution while this property is lacking or less pronounced in crystalloids. A brief quotation from a paper by Graham will illustrate these definitions:

"Among the latter [*i.e.*, the substances with low order of diffusibility] are hydrated silicic acid, hydrated alumina, and other metallic peroxides of the aluminous class, when they exist in the soluble form; and starch, dextrin and the gums, caramel, tannin, albumen, gelatine, vegetable and animal extractive matters. Low diffusibility is not the only property which the bodies last enumerated possess in common. They are distinguished by the gelatinous character of their hydrates. Although

often largely soluble in water, they are held in solution by a most feeble force. They appear singularly inert in the capacity of acids and bases, and in all the ordinary chemical relations.[1] But, on the other hand, their peculiar physical aggregation with the chemical indifference referred to, appears to be required in substances that can intervene in the organic processes of life. The plastic elements of the animal body are found in this class. As gelatine appears to be its type, it is proposed to designate substances of the class as *colloids*, and to speak of their peculiar form of aggregation as the *colloidal condition of matter*. Opposed to the colloidal is the crystalline condition. Substances affecting the latter form will be classed as *crystalloids*. The distinction is no doubt one of intimate molecular constitution."[2]

It is therefore obvious that there are according to Graham at least two essential differences between colloids and crystalloids, the difference in diffusion through membranes, and the difference in the tendency to form aggregates in solutions. We shall see in this volume that the chief if not all the characteristics of colloidal behavior can be explained mathematically from the difference in diffusibility between colloids and crystalloids, while the tendency of the protein molecules to form aggregates plays only an indirect role, namely, by immobilizing one kind of ions without interfering with the mobility of other ions.

In modern colloid chemistry it has, however, become customary to consider the tendency of colloids to form aggregates as the fundamental property, for the reason that the precipitation of colloids was the chief topic of research and discussion in colloid chemistry, and precipitation is, of course, due to the formation of aggregates. The colloidal state is defined by colloid chemists as that state of matter in which the ultimate units in solutions are no longer isolated molecules or ions, but aggregates of molecules for which Naegeli had introduced the term micella (small crumb). Thus Zsigmondy states,

"that the essential and characteristic constituents of colloidal solutions are very small ultramicroscopic particles the dimensions of which lie between molecular and microscopic size. . . . These ultramicroscopic

[1] This is no longer correct, as we shall see.

[2] Graham, T., *Phil. Trans.*, pp. 183–224, 1861. Reprinted in "Chemical and Physical Researches," p. 553, Edinburgh, 1876.

particles (ultramicrons) have the same significance for colloidal solutions as the isolated molecules have for crystalloidal solutions."[1]

The idea that the ultimate unit of the colloidal solution is not the molecule or ion of the solute but an aggregate induced colloid chemists to propose a new type of chemistry in which the laws of classical chemistry were replaced by laws peculiar to colloid chemistry. It seemed improbable to them that the stoichiometrical laws of classical chemistry should hold for colloidal solutions in which the ultimate units were larger aggregates of molecules, since they argued that only the surface of such aggregates should be capable of reacting with other substances. The stoichiometrical relations valid in classical chemistry were as a consequence replaced in colloid chemistry by an empirical formula, Freundlich's so-called adsorption formula, which was supposed to account for surface action.[2] Recent investigations by Langmuir[3] have furnished the proof that Freundlich's adsorption formula does not hold for the reaction of gases with mica, glass, and platinum possessing a smooth surface, and Langmuir was able to show that the forces which act in these cases are the purely chemical forces of primary or secondary valency. Like most empirical formulas the adsorption formula may hold within a limited range of observations, but not throughout the whole range of variation, and Langmuir states that this was also true for the adsorption formula in his experiments.

John A. Wilson and Wynnaretta H. Wilson[4] have made a most important contribution towards the question of the applicability of the adsorption formula to colloidal problems, in which they were also led to a rejection of the adsorption formula and to the adoption of a purely chemical interpretation. Their discussion is based on the experiments of Procter and Wilson on gelatin and the facts to be given in this book fully support their skeptical attitude towards the adsorption formula.

Even if we assume that the protein solutions contain no free protein ions or molecules—which is contradicted by the experi-

[1] Zsigmondy, R., "Kolloidchemie," 2nd ed., Leipsic, 1918.
[2] Freundlich, H., "Kapillarchemie," Leipsic, 1909.
[3] Langmuir, I., *J. Am. Chem. Soc.*, vol. 40, p. 1361, 1918.
[4] Wilson, J. A. and Wilson, W. H., *J. Am. Chem. Soc.*, vol. 40, p. 886, 1918.

ments on potential difference and osmotic pressure to be discussed later—such an assumption does not lead to the idea that chemical reactions occur only at the surface of the micellæ for the simple reason that solid gels of proteins (*e.g.*, of gelatin) are easily permeable to acids, alkalies, and salts or to crystalloids in general. Chemical reactions are, therefore, not restricted to the surface of protein micellæ.

While a number of authors, like Bugarszky and Liebermann,[1] Osborne,[2] Robertson,[3] Pauli,[4] and others assumed that the reactions of proteins are purely chemical, this assumption could not be proved conclusively until the modern methods of measuring the hydrogen ion concentration of protein solutions were developed by Friedenthal, Sørensen,[5] Michaelis,[6] Clark,[7] and their collaborators. On the basis of these methods it was easy to demonstrate the purely stoichiometrical character of the combination of proteins with acids and alkalies.

Thus it was proved that gelatin combines with acids only when the hydrogen ion concentration of the solution is above a certain critical point, namely greater than N/50,000 (or pH = 4.7).[8] At hydrogen ion concentrations above N/50,000, H_3PO_4 dissociates as a monobasic acid. Hence, if gelatin combines stoichiometrically with acids it should require three times as many cubic centimeters of 0.1 N H_3PO_4 as it requires cubic centimeters of 0.1 N HCl or HNO_3 to bring 1 gm. of gelatin in 100 cc. solution from a hydrogen ion concentration of N/50,000 to that of, *e.g.*, N/1,000. The strong acid H_2SO_4 dissociates, however, in this

[1] Bugarszky, S. and Liebermann, L., *Arch. ges. Physiol.*, vol. 72, p. 51, 1898.

[2] Osborne, T. B., Die Pflanzenproteine: *Ergeb. Physiol.*, vol. 10, p. 47, 1910.

[3] Robertson, T. B. "The Physical Chemistry of the Proteins," New York, London, Bombay, Calcutta, and Madras, 1918.

[4] Pauli, W., *Fortschr. naturwiss. Forschung*, vol. 4, p. 223, 1912. "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

[5] Sørensen, S. P. L., see Bibliography given in W. M. Clark, "The Determination of Hydrogen Ions," Baltimore, 1920.

[6] Michaelis, L., "Die Wasserstoffionenkonzentration," Berlin, 1914.

[7] Clark, W. M., "The Determination of Hydrogen Ions," Baltimore, 1920.

[8] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920–21. *Science*, vol. 52, p. 449, 1920. *J. chim. physique*, vol. 18, p. 283, 1920.

range of hydrogen ion concentration as a dibasic acid and hence, it should require as many cubic centimeters of 0.1 N H_2SO_4 as it requires cubic centimeters of 0.1 N HCl to bring the same 1 per cent solution of gelatin from a hydrogen ion concentration of N/50,000 to one of N/1,000. Titration experiments proved the correctness of these and similar conclusions, not only in the case of gelatin but also of other proteins, thus leaving no doubt that proteins combine with acids or alkalies according to the stoichiometrical laws of general chemistry.[1]

It was merely an unfortunate historical accident that the colloidal behavior of proteins was investigated before the convenient methods of measuring the hydrogen ion concentration were developed; otherwise, we should probably never have heard of the idea that the chemistry of colloids differs from the chemistry of crystalloids, at least as far as the proteins are concerned. It was this methodical error of not measuring the hydrogen ion concentration of colloidal solutions and of gels which prevented the development of an exact theory of colloidal behavior and which gave rise to the statement of Zsigmondy quoted in the preface.

The reason that measurements of the hydrogen ion concentration are paramount for the understanding of the chemical and physical behavior of the proteins lies in the fact that proteins are amphoteric electrolytes capable of forming ionizable salts with acids as well as with alkalies, according to the hydrogen ion concentration. When the hydrogen ion concentration exceeds a certain critical value (which varies for different proteins) the protein behaves as if it were a base, like NH_3, capable of forming salts with acids; while when the hydrogen ion concentration of the solution is below this critical value the protein behaves as if it were a fatty acid, *e.g.*, CH_3COOH, capable of forming salts with bases. At the critical value of the hydrogen ion concentration the protein can practically combine neither with an acid nor a base nor a neutral salt.[2] This critical hydrogen ion concentration is called the "isoelectric" point of the protein. Moreover, we shall see that the fraction of 1 gm. of originally isoelectric

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, pp. 85, 547, 1920–21.

[2] Loeb, J., *J. Gen. Physiol.*, vol. 1, pp. 39, 237, 1918–19. *Science*, vol. 52, p. 449, 1920, *J. chim. physique*, vol. 18, p. 283, 1920.

protein in 100 c.c. solution capable of combining with an acid or alkali, is also a definite function of the hydrogen ion concentration.

2. The Isoelectric Point of Proteins

The conception of the "isoelectric point" of proteins was introduced before its chemical meaning was recognized and it attracted attention because it was connected with the precipitation of colloids, a phenomenon on which the interest of a number of investigators had been focussed. The conception of the isoelectric point of proteins, which is due to W. B. Hardy,[1] must be considered as the starting point for the physical chemistry of proteins. This author found in 1899 that white of egg diluted with eight or nine times its volume of distilled water, filtered, and boiled when put into an electrical field migrated in an opposite direction according to whether the reaction of the fluid was acid or alkaline. When the fluid had an alkaline reaction, the particles moved in an electrical field from the cathode to the anode; when the fluid was acid, the direction of the motion of the particles was the reverse, namely, from the anode to the cathode; when the fluid was neutral the movement of the particles under the influence of a current was so slight that it was difficult to detect.

"I have shown that the heat-modified proteid is remarkable in that its direction of movement [in an electric field] is determined by the reaction acid or alkaline, of the fluid in which it is suspended. An immeasurably minute amount of free alkali causes the proteid particles to move against the stream while in presence of an equally minute amount of free acid the particles move with the stream. In the one case therefore the particles are electro-negative, in the other they are electro-positive. Since one can take a hydrosol in which the particles are electro-negative and, by the addition of free acid, decrease their negativity, and ultimately make them electro-positive it is clear that there exists some point at which the particles and the fluid in which they are immersed are isoelectric.

"The isoelectric point is found to be one of great importance. As it is neared the stability of the hydrosol diminishes until, at the isoelectric point, it vanishes, and coagulation or precipitation occurs, the one or the other according to whether the concentration of the proteid is high or

[1] Hardy, W. B., *Proc. Roy. Soc.*, vol. 66, p. 110, 1900.

low, and whether the isoelectric point is reached slowly or quickly, and without or with mechanical agitation."

In a preliminary note[1] on his work on globulins published in 1903 Hardy gives an interpretation of the influence of H and OH ions on the direction of migration of protein particles in an electrical field which was destined to play an important role in colloid chemistry, since it suggested to the later workers that the H and OH ions produced their influence on the electrical charge of the protein particles through a process of adsorption.

"The properties of globulins in solution seem to justify the following view: *They are not embraced by the theorem of definite and multiple proportions. Therefore they are conditioned by purely chemical forces only in a subsidiary way.* A precipitate of globulin is to be conceived not as composed of molecular aggregates but of particles of gel. I have shown elsewhere that gelation and precipitation of colloidal solutions are continuous processes. These particles of gel when suspended in a fluid containing ions are penetrated by those ions. Let the fundamental assumption be that the higher the specific velocity of an ion the more readily it will become entangled within the colloidal particle. Then as H and OH ions have by far the highest specific velocity the colloidal particle will entangle an excess of H ions in acid and thereby acquire a + charge and of OH ions in alkali and thereby acquire a − charge. These charges will decrease the surface energy of the particle and thereby lead to changes in their average size."

Perrin adopted the idea that H and OH ions confer their electrical charge to colloidal particles on account of their relatively large velocity of migration, whereby they were readily adsorbed by the colloidal particle. The hypothesis of a preferential adsorption of H and OH ions by colloidal particles has since played a great role in colloid chemistry.

In 1904 the writer of this volume offered instead of this colloidal a purely chemical view of the significance of the isoelectric point and of the cause of the influence of acids and alkalies on the direction of the migration of the colloidal particles.[2]

"It seems to the writer, however, that a different view of these phenomena is possible whereby they appear in harmony with the view of electrolytic origin of the charges of colloids. The proteids are known

[1] Hardy, W. B., *J. Physiol.*, vol. 29, p. 29, 1903.

[2] Loeb, J., *Univ. of Cal. Publications, Physiology*, vol. 1, p. 149, 1904.

to be amphoteric in their reaction. If they be slightly dissociable they will send H as well as OH ions into the solution. When the particles send more H ions than OH ions into the solution they will have a negative charge while they will have a positive charge when more OH ions are given off than H ions. If acid is added to the solution in sufficient concentration the amphoteric colloidal particle will send more OH ions into the solution than H ions and hence, will assume a positive charge. The reverse will be the case in an alkaline solution. It harmonizes with this idea that, as Hardy found, neutral salts do not influence the sign of the electrical charge of the globulins."

We shall see later on that this suggestion explains the source of the electrical charge of isolated protein ions but explains only indirectly the charge of larger aggregates.

In his famous paper on "Colloidal Solution" published in 1905, Hardy[1] abandons the physical view which he expressed in 1903 and adopts "a frankly chemical standpoint."

"Globulin therefore is an amphoteric substance and its acid function is much stronger than its basic function. As an acid it is strong enough to form salts readily with bases so weak as aniline, glycocoll, and urea; acting as a base it forms salts with weak acids, such as acetic, and boracic acids, which are very unstable in presence of water."

While Hardy accepts the idea of an electrolytic origin of the charges of proteins, he does not seem to be ready to concede that the reactions of proteins with acids and alkalies are purely stoichiometric, as the following quotations indicate.

"Though one may speak of the colloid particles as being ionic in nature they are sharply distinct from true ions in the fact that they are not of the same order of magnitude as are the molecules of the solvent, the electric charge which they carry is not a definite multiple of a fixed quantity and one cannot ascribe to them a valency, and their electrical relations are those which underlie the phenomena of electrical endosmose. To such ionic masses I would give the name 'pseudo-ions' and I propose to treat globulin solutions from the standpoint of a hypothesis of 'pseudo-ions.'[2]

And in 1910 Wood and Hardy[3] express the view that proteins

[1] Hardy, W. B., *J. Physiol.*, vol. 33, p. 251, 1905–06. See also, Hardy, W. B., *Proc. Roy. Soc.*, vol. 79, p. 413, 1907.

[2] Hardy, W. B., *J. Physiol.*, vol. 33, pp. 256–257, 1905–06.

[3] Wood, T. B. and Hardy, W. B., *Proc. Roy. Soc.*, vol. 81, p. 38, 1909.

"react with acids and alkalies to form salts, but the reactions are not precise, an indefinite number of salts of the form $(B)_nBHA$ being formed where the value of n is determined by conditions of temperature and concentration, and of inertia due to electrification of internal surfaces within the solution."

There are two elements in this view which should be separated. The suggestion that the electrical charges of the micellæ are not "a definite multiple of a fixed quantity" harmonizes with the results to be given later. The other suggestion, however, "that the reactions are not precise" seems to be contradicted by the stoichiometrical facts to be enumerated in the fourth chapter.

When the methods of measuring the hydrogen ion concentration had been developed by H. Friedenthal and by Sørensen it became possible to determine the isoelectric point of genuine proteins. This was first done by Michaelis and his collaborators in 1910. Michaelis used the same method of migration of the particles in an electrical field which had been used by Hardy. The isoelectric point is, according to Michaelis, that hydrogen ion concentration at which the particles migrate neither to the anode nor to the cathode. The following figures give the hydrogen ion concentrations defining the isoelectric points of different proteins as determined by Michaelis.[1]

Genuine serum albumin	2×10^{-5}N
Genuine serum globulin	4×10^{-6}N
Oxyhemoglobin	1.8×10^{-7}N
Gelatin	2×10^{-5}N
Casein	2×10^{-5}N

According to Sørensen the isoelectric point of crystalline egg albumin is near that of serum albumin (namely, at a pH of 4.8).[2]

We shall denote in this book the hydrogen ion concentration by Sørensen's logarithmic symbol pH; *e.g.*, the concentration $2 \times 10^{-5}\text{N} = 10^{-4.7}\text{N}$ is written merely pH 4.7, the minus sign being omitted.

If we assume that the ultimate units of a protein solution are as a rule isolated protein molecules or ions which react stoichi-

[1] MICHAELIS, L., "Die Wasserstoffionenkonzentration," p. 54 ff, Berlin, 1914.

[2] SØRENSEN, S. P. L., Studies on proteins: *Compt. rend. trav. Lab. Carlsberg*, vol. 12, Copenhagen, 1915–17.

ometrically with acids and alkalies, forming highly dissociable metal proteinates or protein-acid salts, we may define the isoelectric point of a protein as that hydrogen ion concentration in which the protein exists practically in a non-ionogenic (or non-ionized) condition being able to form practically neither metal proteinate nor protein-acid salt. We shall see that this theoretical result leads to a simple practical method of preparing proteins entirely or practically free from ionogenic impurities.

The fact that solutions and suspensions of proteins are least stable at the isoelectric point is then connected with the purely chemical fact that proteins are amphoteric electrolytes which exist at their isoelectric point in the form of practically non-ionizable protein molecules.

3. The Adsorption Theory and the Precipitation of Proteins

The interest of most investigators of colloidal phenomena was centered on the precipitation of colloids, especially in those cases where the precipitation required low concentrations of electrolytes. The explanation accepted by the majority of authors is based on the assumption of an adsorption of ions by the colloid.

Hardy explained his discovery that proteins are most easily flocculated from their solutions at the isoelectric point by the fact that at that point the electrical charges of the protein particles are a minimum, a conclusion derived from his observation that at the isoelectric point proteins do not migrate in an electrical field. He concluded from this that the stability of colloidal solutions is due to the potential difference between each colloidal particle and the surrounding liquid. In this state the charged particles must repel each other with the result that they become evenly distributed through the solvent. When the charge is annihilated or sufficiently diminished "the adhesion or 'idio-attraction' as Graham called it, of the colloid particles for each other makes them cohere where they come together."[1] He originally assumed the positive charge of the particles in the acid solution to be due to a preferential adsorption of H ions and the negative charge in the presence of alkali to the adsorption

[1] Wood, T. B. and Hardy, W. B., *Proc. Roy. Soc.*, vol. 81, p. 41, 1909.

of OH ions. Later he abandoned this view, which, however, is still held by many chemists.

Another explanation of the coalescence of the particles which have lost their electrical charge was given by Bredig on the basis of surface tension changes. The surface tension at the boundary of a micella and water is diminished when the particles are electrically charged and reaches a maximum when the charge is annihilated. Since at the isoelectric point the electrical charges of the particles are nil the surface tension at the boundary of particles and water must be a maximum and as a consequence two isoelectric particles upon coming in contact are forced to coalesce; while the particles will not coalesce when the surface tension is low.[1]

It is, however, doubtful whether the coalescence of the non-charged colloidal particles is due to surface tension effects. Zsigmondy[2] points out that Powis'[3] observations on the precipitation of droplets of oil emulsion by salts make it more probable that the coalescence is due to forces of attraction between the droplets, since in commencing flocculation the individual oil globules only adhere to each other without coalescing into larger droplets.

Colloids can, however, be flocculated by salts even if their solution is not at the isoelectric point. In this case Hardy assumes that the addition of the salt lowers the potential difference between the colloidal particle and the solvent. Schulze, Linder and Picton, as well as Hardy[4] had found that the ion which is responsible for the flocculation has always the opposite sign of charge to the colloidal particle, and moreover, that the coagulative power of the ion increases rapidly with its valency.[5] This rule was considered to strengthen the adsorption theory.

It was assumed that the micellæ possess an electrical charge

[1] Michaelis, L., "Die Wasserstoffionenkonzentration," pp. 49–50, Berlin, 1914.

[2] Zsigmondy, R., "Kolloidchemie," 2nd ed., p. 63, Leipsic, 1918.

[3] Powis, F., *Z. physik. Chem.*, vol. 89, pp. 91, 179, 186, 1915.

[4] Hardy, W. B., *Proc. Roy. Soc.*, vol. 66, p. 110, 1900, *J. Physiol.*, vol. 33, p. 251, 1905–06.

[5] For the details and the literature see Burton, E. F., "The Physical Properties of Colloidal Solutions," 2nd ed., London, New York, Bombay, Calcutta, and Madras, 1921.

which will cause them to "adsorb" most readily those ions of an electrolyte which have the opposite sign of charge from the colloidal particle. This adsorption is supposed to annihilate the charge of the particles causing them to coalesce. The higher the charge of the ion the more readily it is adsorbed; and this is presumed to explain why the flocculating action of ions increases with their valency.[1]

The hypothesis that the electrical charges of micellæ of proteins are diminished or annihilated by the preferential adsorption of the ions of a salt rests on no measurements and the hypothesis has never advanced beyond the stage of vague qualitative speculation. Such speculations would never have been accepted or considered if it were not for the fact that there existed no direct measurements of the charges of suspended protein particles. The writer found a method of directly measuring the P.D. between protein particles and surrounding liquid, and was thus able to follow minutely the influence of the hydrogen ion concentration and of the addition of salts on the P.D.[2] The quantitative data thus gained made it possible to investigate the origin of the P.D. and it was found that this P.D. is due to the fact that proteins form ionizable salts with acids and bases. Whenever protein ions are prevented from diffusing through membranes or gels permeable to crystalloidal ions, peculiar equilibrium conditions are established resulting in an unequal distribution of the oppositely charged crystalloidal ions between colloidal particle and surrounding liquid. This unequal distribution of oppositely charged ions leads to the P.D. at the boundary of micellæ and surrounding liquid. It is possible to explain mathematically, from Donnan's equation for such membrane equilibria, the influence of acids, alkalies, and neutral salts on the charges of the micellæ, and it can be shown that the observed P.D. agrees quantitatively with that calculated from the equilibrium equation. It thus turns out that the explanation of the annihilation of the

[1] For a full presentation of the adsorption theory the reader is referred to BANCROFT, W. D., "Applied Colloid Chemistry," New York, London, 1921, in this series, and to LEWIS, W. C. MCC., "A System of Physical Chemistry," 2nd ed., vol. 1, p. 346, London, New York, Bombay, Calcutta, and Madras, 1920.

[2] LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 667, 1920–21; vol. 4, p. 351, 1921–22.

charges of micellæ by neutral salts depends on the fact that proteins combine stoichiometrically with acids and alkalies forming true ionizable salts. The agreement between calculated and observed values is so close that there is neither any need nor room for speculations on adsorption, unless it can be shown that the adsorption hypothesis furnishes an equally good mathematical and quantitative agreement between observed and calculated P.D.

4. The Hofmeister Ion Series

Hofmeister[1] was the first to investigate the effects of different salts on the physical properties of proteins. He and his followers observed that the relative effects of anions on the precipitation, the swelling, and other properties of proteins seemed very definite and that the anions could be arranged apparently in definite series according to their relative efficiency, the order being independent of the nature of the cation. Similar series were also found for the cations, though these series seemed to be less definite. These Hofmeister series were a puzzle to those who accepted the chemical viewpoint of the behavior of proteins, inasmuch as it was impossible to discover in these series a relation to the typical combining ratios of the ions.

To illustrate this we will quote the order which, according to Pauli,[2] represents the relative efficiency of different acids on the viscosity of blood albumin,

$$\text{HCl} > \text{monochloracetic} > \text{oxalic} > \text{dichloracetic} > \text{citric} > \text{acetic} > \text{sulphuric} > \text{trichloracetic acid},$$

where HCl increased the viscosity most and trichloracetic or sulphuric least. In this series the strong monobasic acid HCl is followed by the weak monochloracetic acid, this is followed by the dibasic oxalic acid; later follows the weak tribasic citric acid, then the very weak monobasic acetic acid, then the strong dibasic sulphuric acid, and finally again a monobasic acid, trichloracetic.

According to Hofmeister, gelatin swells more in chlorides,

[1] Hofmeister, F., *Arch. exp. Path. u. Pharm.*, vol. 24, p. 247, 1888; vol. 25, p. 1, 1888–89; vol. 27, p. 395, 1890; vol. 28, p. 210, 1891.

[2] Pauli, W., *Fortschr. naturwiss. Forschung*, vol. 4, p. 223, 1912.

bromides, and nitrates than in water, while in acetates, tartrates, citrates, or sugar it swells less than in water. R. S. Lillie[1] arranges ions according to their depressing effect on the osmotic pressure of gelatin solution in the following way:

$$Cl > SO_4 > NO_3 > Br > I > CNS$$

These series[2] again betray no relation to the stoichiometrical properties of the ions. As long as these Hofmeister ion series were believed to have a real existence it seemed futile to decide for or against a purely chemical theory of the behavior of colloids since even with a bias in favor of a chemical theory the Hofmeister series remained a riddle.

The writer believes that he has removed these difficulties by using protein solutions of equal hydrogen ion concentration as the standard of comparison.

In this way it was found that a number of authors had erroneously attributed the effects of an alteration of the hydrogen ion concentration upon the physical properties of a protein to a difference in the specific action of the anion or cation added. Thus it was always believed that acetates have almost as great a "dehydrating" action as sulphates, but it was overlooked that acetic acid is a weak acid, and that in the experiments referred to the authors failed to compare the effects of SO_4 and CH_3COO at the same hydrogen ion concentration. When this error is avoided it can be shown that acetates influence the swelling, osmotic pressure, and viscosity of protein solutions in the same way as chlorides or nitrates, but not in the same way as sulphates; in other words, anions of the same valency act alike.[3]

By taking into consideration the hydrogen ion concentration it was possible to show that the assumption of specific differences in the action of different ions of the same valency and sign of charge was due to a methodical error; and that the Hofmeister rule must be replaced by a simple valency rule, according to which only the valency and sign of charge of an ion influence the colloidal behavior of a protein but that the other properties of

[1] Lillie, R. S., *Am. J. Physiol.*, vol. 20, p. 127, 1907–08.

[2] A fuller discussion of these series is found in Höber, R., "Physikalische Chemie der Zelle und der Gewebe," Leipsic and Berlin, 1914.

[3] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 391, 1920–21.

the ion have no influence as long as no constitutional change in the protein molecule occurs.

This fact established a complete harmony between the results of the titration experiments and the influence of ions on the physical properties of gelatin. In the titration experiments it had been found that at a hydrogen ion concentration of above 2×10^{-5}N weak dibasic or tribasic acids generally combine with a protein as if they were entirely or chiefly monobasic. Hence, the anions of the protein salts formed with these weak dibasic or tribasic acids, *e.g.*, phosphoric, citric, tartaric, succinic, were monovalent, and it was found that the osmotic pressure or viscosity of solutions of protein phosphates were the same as those of protein chlorides for the same hydrogen ion concentration and the same concentration of originally isoelectric protein.

On the other hand, the titration experiments showed that the anion of protein sulphate is dibasic and it was found that the osmotic pressure and viscosity of protein sulphate is less than one-half of that of protein chloride or phosphate or succinate, etc., at the same hydrogen ion concentration and the same concentration of originally isoelectric protein.[1]

In this way the influence of ions on the physical properties of proteins, especially in the case of gelatin, turned out to be in harmony with the results of titration experiments. In the case of gelatin and apparently also crystalline egg albumin, only the valency but not the nature of the ion in combination with the protein influences its properties. The statements to the contrary were due to two errors, first and foremost, the failure to measure the hydrogen ion concentration of the protein solutions, and second, the confusion of phenomena of solubility with phenomena of colloidal behavior.

5. The Aggregation Hypothesis

It was perhaps not very fortunate for the development of a theory of colloids that the attention of investigators was focussed especially on the phenomena of precipitation. Since precipitation is due to an aggregation of particles it over-emphasized the significance of aggregate formation. This led, as we have seen, to the erroneous idea that proteins do not combine stoichiome-

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, pp. 85, 247, 1920–21.

trically with other compounds, since aggregates were assumed to react only at their surface—an assumption which, as already stated, is not warranted in the case of proteins, since protein gels are freely permeable to crystalloids. It led, however, to another equally fatal idea, that this aggregate formation would explain all the colloidal phenomena. Thus when R. S. Lillie[1] made the important observation that neutral salts depress the osmotic pressure of gelatin solutions, it seemed natural to explain this fact from the precipitating action of salts, by assuming that the addition of salt caused an aggregation of gelatin molecules or ions into larger aggregates. This would lead to a diminution of the number of particles in solution. But it was also found that the addition of salts depresses the viscosity of protein solutions and the swelling of solid proteins. We shall see later that the formation of aggregates out of isolated protein molecules or ions increases the viscosity of a gelatin solution.[2] Hence, if the addition of a salt to a protein solution diminishes its osmotic pressure by causing an increased formation of aggregates the same addition of salt should increase the viscosity of such a solution. The reverse, however, happens, the viscosity of the solution being decreased by the addition of salt.

There is nevertheless a connection between the phenomena of precipitation and the depressing effect of salts on viscosity, osmotic pressure, and swelling of proteins. Schulze, Linder and Picton, and Hardy had observed that in the precipitation of colloids that ion is active which has the opposite sign of charge from the protein particle, and that the efficiency of the active ion increased with its valency. The same rule applies to the depressing action of salts on the osmotic pressure, viscosity, and swelling of proteins. By trying to explain these latter effects from the precipitating action of salts the colloid chemists put the cart before the horse, and were led into a hopeless contradiction with the facts. We shall see that by taking the reverse step, namely, of explaining the precipitating action of salts from their depressing action on osmotic pressure and P.D. of protein solutions, everything becomes clear and consistent. But this step could not be taken as long as the belief in the adsorption theory

[1] LILLIE, R. S., *Am. J. Physiol.*, vol. 20, p. 127, 1907–08.
[2] LOEB, J., *J. Gen. Physiol.*, vol. 4, p. 97, 1921–22.

of colloids prevailed. The quantitative explanation of the colloidal behavior of proteins to be given in this book rests on the proof that they form true ionizable salts with acids and alkalies.

6. Pauli's Hydration Theory

Laqueur and Sackur,[1] in studying the influence of the addition of different quantities of NaOH to a given mass of casein, assumed correctly that the two substances combined to form sodium caseinate. The viscosity of the sodium caseinate solution was high and it varied in a peculiar way with the quantity of NaOH added to the casein. When little NaOH was added, the viscosity increased at first with an increase in the quantity of the NaOH added until a maximum was reached, when the addition of more NaOH diminished the viscosity again. This again is a fundamental fact which has since been confirmed for the influence of acids and alkalies not only upon the viscosity but also upon other properties of proteins and which holds not only for casein but apparently for all proteins.

Laqueur and Sackur explained their results on the basis of Reyher's[2] experiments on the viscosity of solutions of fatty acids. Reyher had found that the viscosity of solutions of salts of the fatty acids is greater than that of solutions of fatty acids themselves; and since the salts of the fatty acids undergo electrolytic dissociation to a much greater extent than the acids it was assumed that the increase in viscosity is determined chiefly by the ionization. Laqueur and Sackur made the same assumption for the casein solutions, attributing the high viscosity of casein solutions to the casein ions, and they support their assumption by the fact that the addition of little NaOH to casein at first increases the viscosity until a maximum is reached and that the addition of more NaOH diminishes the viscosity again. A diminution of viscosity could also be produced by the addition of neutral salt to the solution of Na caseinate. Laqueur and Sackur assume that this drop in the viscosity is caused by a lowering of the degree of electrolytic dissociation of the Na caseinate by the Na ion of the NaOH or NaCl added in excess.

[1] Laqueur, E. and Sackur, O., *Beitr. chem. Physiol. u. Pathol.*, vol. 3, p. 193, 1903.

[2] Reyher, R., *Z. physik. Chem.*, vol. 2, p. 744, 1888.

The idea that the viscosity of protein solution depends primarily upon the protein ion was accepted by W. Pauli,[1] who made the additional hypothesis that each protein ion is hydrated; *i.e.*, that each individual protein ion is surrounded by a considerable shell of water. Pauli worked with blood albumin which had been freed from salts by a dialysis continued for several weeks. When he added acid to water-soluble albumin, the viscosity increased first from 1.0623 for the pure albumin solution to 1.2937 when the concentration of HCl added to the albumin solution was 0.017 N. When the HCl concentration was increased to 0.05 N the viscosity was only 1.1667. The following figures give the data according to Pauli:

Concentration of HCl	0.0 N	0.005 N	0.01 N	0.012 N	0.017 N	0.02 N	0.03 N	0.04 N	0.05 N
Viscosity	1.0623	1.2555	1.233	1.274	1.2937	1.2770	1.224	1.1822	1.1667

Pauli assumed that the protein ions are surrounded by a jacket of water, while the non-ionized molecules of protein he assumed not to be hydrated. Addition of a little HCl to isoelectric albumin would cause the transformation of non-ionized albumin into albumin chloride which is highly ionized and hence assumed to be highly hydrated; the more acid is added the more albumin chloride and the more hydrated albumin ions should be formed. Hence, the viscosity should at first increase with the quantity of acid added, until a point is reached where the addition of more acid represses the degree of electrolytic dissociation of the albumin chloride on account of the high concentration of the Cl ion common to both protein chloride and HCl.

If we intend to use these ideas for the explanation of the influence of the valency of ions on the physical properties of proteins we are compelled to assume that the degree of electrolytic dissociation of gelatin salts with bivalent ions is lower than that of gelatin salts with monovalent ions. Since, *e.g.*, the viscosity of gelatin chloride solutions is considerably higher than the viscosity of gelatin sulphate solutions of the same hydrogen ion concentration and the same concentration of originally isoelectric gelatin, we should have to conclude that the degree of electrolytic dissociation of gelatin sulphate is considerably less than that of gelatin chloride.

[1] Pauli, W., *Fortschr. naturwiss. Forschung*, vol. 4, p. 223, 1912; "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

The writer put this theory to a test by measuring the electrical conductivity of solutions of different gelatin salts at different pH, with the result that the parallelism between the concentration of protein ions and the physical properties of proteins demanded by Pauli's theory could not be demonstrated (see Chap. VII). Lorenz,[1] Born,[2] and other authors have recently reached the conclusion that the idea of a hydration of ions is not tenable in the case of polyatomic ions.[3]

The increase in viscosity of certain protein solutions through the addition of acid or alkali to isoelectric proteins is caused by the ionization of proteins, but the connection is not the direct one suggested by Laqueur and Sackur but an indirect one due to the role of protein ions in the establishment of a Donnan equilibrium.

7. Donnan's Membrane Equilibrium

With the proof of the stoichiometrical character of the combination of proteins with acids and alkalies the explanation of colloidal behavior on the basis of the adsorption theory became untenable and another theoretical basis had to be found. The explanation offered in this volume is based on Donnan's theory of membrane equilibria.

Donnan[4] has shown that when a membrane separates two solutions of electrolytes one of which contains one ion which cannot diffuse through the membrane while all the other ions can diffuse through the membrane, the result will be an unequal distribution of the diffusible ions on the opposite sides of the membrane. At equilibrium the products of the concentrations of each pair of oppositely charged diffusible ions are the same on the opposite sides of the membrane. This unequal concentration of the crystalloidal ions must give rise to potential differences

[1] Lorenz, R., *Z. Elektrochem.*, vol. 26, p. 424, 1920.

[2] Born, M., *Z. Elektrochem.*, vol. 26, p. 401, 1920.

[3] The term "hydration" is often used in colloid chemistry in a vague way to designate such phenomena as the swelling of proteins which is a purely osmotic phenomenon. It is obvious that it can only lead to confusion if the term hydration is used for osmotic pressure. In this volume the term hydration is only used in the sense of Kohlrausch and Pauli.

[4] Donnan, F. G., *Z. Elektrochem.*, vol. 17, p. 572, 1911.

and osmotic forces, and we intend to show that these forces furnish the explanation of colloidal behavior.

It may be best to quote Donnan's theory in his own words:

"We suppose that the membrane (indicated in the following diagram by a vertical line) be impermeable for the anion R of a salt NaR (and also for the non-dissociated part of the salt NaR), but permeable for all the other ions and salts to be considered in this connection . . .

"Suppose that in the beginning we have a solution of NaR on one side of the membrane (indicated by a vertical line) and of NaCl on the other side

$\overset{+}{\mathrm{Na}}$	$\overset{+}{\mathrm{Na}}$
$\overset{-}{\mathrm{R}}$	$\overset{-}{\mathrm{Cl}}$
(1)	(2)

In this case NaCl will diffuse from (2) to (1). In the end the following equilibrium will result:

$\overset{+}{\mathrm{Na}}$	$\overset{+}{\mathrm{Na}}$
$\overset{-}{\mathrm{R}}$	
$\overset{-}{\mathrm{Cl}}$	$\overset{-}{\mathrm{Cl}}$
(1)	(2)

"When this equilibrium is established the energy required to transport reversibly and isothermally 1 grammolecule $\overset{+}{\mathrm{Na}}$ from (2) to (1) equals the energy which can be gained by the corresponding reversible and isothermal transport of a grammolecule $\overset{-}{\mathrm{Cl}}$. In other words, we consider the following infinitely small isothermal and reversible change of the system:

$$\left\{\begin{array}{l} \delta n \text{ Mol } \overset{+}{\mathrm{Na}} \text{ (2)} \rightarrow \text{(1)} \\ \delta n \text{ Mol } \overset{-}{\mathrm{Cl}} \text{ (2)} \rightarrow \text{(1)} \end{array}\right\}$$

"The energy which can be gained in this way (*i.e.*, the diminution of free energy) is zero, hence:

$$\delta n \cdot \mathrm{RT} \log \frac{[\overset{+}{\mathrm{Na}}]_2}{[\overset{+}{\mathrm{Na}}]_1} + \delta n \cdot \mathrm{RT} \log \frac{[\overset{-}{\mathrm{Cl}}]_2}{[\overset{-}{\mathrm{Cl}}]_1} = 0$$

or

$$[\overset{+}{\mathrm{Na}}]_2 \cdot [\overset{-}{\mathrm{Cl}}]_2 = [\overset{+}{\mathrm{Na}}]_1 \cdot [\overset{-}{\mathrm{Cl}}]_1 \qquad (1)$$

where the brackets signify molar concentrations."

This last equation is the equilibrium equation which states that the product of the concentrations of a pair of diffusible cations and anions on one side of the membrane is equal to the product of the concentrations of the same pair of diffusible anions and cations on the other side. Since on the side of the non-diffusible (protein) anion the concentration of cations Na is the sum of the cations in combination with the non-diffusible anion plus the cations in combination with the Cl, while on the other side of the membrane the concentration of the Na ions is only that of Na in combination with Cl and equal to the concentration of Cl, it is obvious that Donnan's equation (1) can only be fulfilled if

$$[\overset{+}{\text{Na}}]_1 > [\overset{+}{\text{Na}}]_2$$

and

$$[\overset{-}{\text{Cl}}]_1 < [\overset{-}{\text{Cl}}]_2$$

This inequality of concentration of the diffusible ions on the opposite sides of the membrane accounts, as we shall see, for the influence of electrolytes on all those properties which colloid chemistry has vainly tried to explain on the basis of the dispersion and hydration hypotheses. The reader will notice that the essential condition determining the equilibrium is the existence of two phases separated by a membrane, one phase containing an ion which cannot diffuse through a membrane which is easily permeable for all the other ions.

This difference in the concentration of the diffusible ions on opposite sides of the membrane must lead to potential differences on opposite sides of the membrane and Donnan shows that this difference must be (on the basis of Nernst's well-known formula)

$$\pi_1 - \pi_2 = \frac{RT}{F} \log \frac{[\overset{+}{\text{Na}}]_2}{[\overset{+}{\text{Na}}]_1} = \frac{RT}{F} \log \frac{[\overset{-}{\text{Cl}}]_1}{[\overset{-}{\text{Cl}}]_2}$$

or since $\frac{RT}{F} = 58$ millivolts (at room temperature) the potential difference on opposite sides of the membrane should be in millivolts

$$\pi_1 - \pi_2 = 58 \log \frac{[\overset{+}{\text{Na}}]_2}{[\overset{+}{\text{Na}}]_1} = 58 \log \frac{[\overset{-}{\text{Cl}}]_1}{[\overset{-}{\text{Cl}}]_2}$$

The writer has tested this consequence of Donnan's theory for solutions of protein salts separated from water by a collodion membrane, with the result that the theory was completely confirmed. Through these measurements of the membrane potentials the correctness of Donnan's theory was proved beyond doubt.

It may be pointed out that it is not necessary that the non-diffusible ion be a colloid; it is only necessary that there be a membrane which prevents one ion from diffusing; it is immaterial whether or not this latter ion be a crystalloid or a colloid. If we had a membrane impermeable for a SO_4 ion but permeable for Na and Cl ions, solutions of NaCl and Na_2SO_4 separated by the membrane would give rise to the Donnan equilibrium, and the Na_2SO_4 solution would probably resemble a solution of Na proteinate in regard to certain features of colloidal behavior, *e.g.*, osmotic pressure and P.D. against water.

Donnan and his collaborators proved the existence of the inequality of the concentration of the diffusible ions of two salt solutions on the opposite sides of a membrane when one of the ions was not able to diffuse through the membrane. Thus Donnan and Allmand investigated

"the distribution of potassium chloride between two compartments separated by a copper ferrocyanide diaphragm, one compartment of which contained potassium ferrocyanide (the membrane being impermeable to the $Fe(CN)_6$ ion). The higher concentration of potassium chloride on the side free from potassium ferrocyanide, and the relation of this unequal distribution to the concentration of the chloride and ferrocyanide, were experimentally established. The results obtained agreed, in general, with the view of membrane equilibria proposed by Donnan, but a discussion of the distribution data combined with electromotive-force measurements appeared to show that, at all events in the case of a copper ferrocyanide membrane and potassium ferrocyanide solutions, the phenomena are not so simple as supposed in the theory."[1]

More recently Donnan and Garner[2] investigated the equilibrium concentration of solutions of Na and K ferrocyanides and of Na and Ca ferrocyanides across a copper ferrocyanide mem-

[1] DONNAN, F. G. and ALLMAND, A. J., *J. Chem. Soc.*, vol. 105, p. 1963, 1914.

[2] DONNAN, F. G. and GARNER, W. E., *J. Chem. Soc.*, vol. 115, p. 1313, 1919.

brane, and the results were in general agreement with Donnan's theory. They also investigated a liquid membrane, namely, amyl alcohol, and the electrolytes employed were KCl and LiCl.

"So far as the preliminary experiments go, the equilibrium concentration of the Li and Cl ions and the undissociated part of the electrolyte agree with Donnan's theory."

We shall see that Donnan's theory explains the influence of electrolytes on the physical properties of proteins. He foresaw the bearing which his theory was likely to have for colloid chemistry and physiology, as is shown by the following remarks.

"In this paper an attempt is made to describe ion equilibria which are bound to occur when certain ions (or their corresponding non-dissociated salt) cannot diffuse through a membrane. Such equilibria possess a great importance for the theory of dialysis and of colloids as well as for the mechanism of the cell and for general physiology."

As far as the writer is aware, Procter and J. A. Wilson were the only authors who attempted the application of Donnan's theory to colloidal problems.

Procter[1] proposed in 1914 an ingenious theory of swelling based on Donnan's membrane equilibrium. According to this theory the force which causes the entrance of water into the gel and thus determines the swelling is the osmotic pressure of the excess of crystalloidal ions inside over that outside the gel, this excess being caused by the Donnan equilibrium. The opposing force which limits the swelling is the force of cohesion of the colloidal particles.

According to Procter, the gelatin ion constituting a jelly of gelatin chloride cannot diffuse and hence can exercise no osmotic pressure, while the chlorine anions in combination with them are retained in the jelly by the electrostatic attraction of the gelatin ion, but exert osmotic pressure. This difference in the diffusibility of the two opposite ions of gelatin chloride gives rise to the establishment of Donnan's membrane equilibrium.

Procter put solid gelatin chloride into a watery solution of HCl and determined by titration the distribution of free HCl inside the gel and outside at the time of equilibrium. In this case there exists inside the gel free HCl and gelatin chloride, out-

[1] Procter, H. R., *J. Chem. Soc.*, vol. 105, p. 313, 1914. Procter, H. R. and Wilson, J. A., *J. Chem. Soc.*, vol. 109, p. 307, 1916.

side HCl. The relative concentration of free HCl inside and outside at the time of equilibrium is determined by the equation for the Donnan equilibrium

$$x^2 = y\,(y + z) \qquad (1)$$

where x is the concentration of the H and Cl ions in the outside solution, y the concentration of H and Cl ions of the free HCl inside the gel, and z the concentration of Cl ions in combination with the gelatin cation. x and y can be determined experimentally and z can be calculated with the aid of the equation. In other words, the distribution of the H and Cl ions on the opposite sides of a membrane is such that the product of the concentrations of the pair of oppositely charged ions is equal in both phases.

"The gelatin salt, like other salts, is highly ionised into the anion and a colloid cation, which either from polymerisation or other causes peculiar to the colloid state cannot diffuse and exerts no measurable osmotic pressure, whilst its anion is retained in the jelly by electrochemical attraction of the colloid ion, but exerts osmotic pressure which, on the one hand, causes the mass to swell with absorption of the external solution, and, on the other, expels a portion of the acid, both anion and hydrion, from this solution absorbed, the result in equilibrium being that the jelly is poorer in hydrion and more concentrated in anion than the external acid solution, the difference of concentration between anion and hydrion in the jelly being, of course, equal to the ionised anion of the gelatin salt, and electrically balanced by the positive gelatin ions; whilst the hydrion concentration in the jelly is less than that of the outer solution by the amount of acid expelled."[1]

By establishing a connection between the volume of the gel and the observed values of x and y, Procter and Wilson were able to calculate the effect of different concentrations of HCl on the swelling of gelatin, and they could show why little acid increased the swelling until a maximum was reached and why the addition of more acid depressed the swelling. They could further show why the addition of neutral salt caused a depression of the swelling.

It is of interest to inquire why this theory of swelling was not accepted and only rarely mentioned in the colloidal literature.

[1] Procter, H. R. and Wilson, J. A., *J. Chem. Soc.*, vol., 109, pp. 309–310, 1916.

In the first place, the application of Donnan's theory to the behavior of proteins requires the proof that proteins form true salts with acids and alkalies and that these salts dissociate electrolytically into a protein ion and a crystalloidal cation or anion. Such an assumption was in conflict with the adsorption hypothesis accepted by the colloid chemists. Moreover, the application of the Donnan theory to proteins tacitly implied that only the valency and sign of charge should have an effect on the proteins, while the nature of the ion should have no effect; and this was in conflict with the belief in the Hofmeister ion series. But even authors, like Robertson, who was a champion of the purely chemical conception of the behavior of proteins, refused to accept Procter's theory of swelling.

"There should be a measurable potential difference between the gelatin jelly and the external medium. This potential difference has been sought for by Ehrenberg who was unable to detect any measurable potential between the interior of a jelly and the external medium."[1]

This gap has been filled by the writer's experiments, which have demonstrated the existence of this potential. The writer has not only been able to furnish support for Procter's theory of swelling but has also been able to show that the potential differences across a membrane separating a solution of a protein salt from pure water fully support Donnan's theory.[2] When we have a solution of a gelatin-acid salt with monovalent anion, *e.g.*, gelatin chloride (or gelatin phosphate) inside a collodion bag which is dipped into pure water, the hydrogen ion concentration as well as the anion concentration on the opposite sides of the membrane are different when osmotic equilibrium is established. The writer was able to show that the potential differences calculated from this difference of the concentration of ions on the basis of Nernst's formula agree with the actually observed P.D., and that the calculated P.D. is the same whether based on a measurement of the difference in the concentration of the hydrogen ions or of the difference in the concentration of the chlorine ions on the opposite sides of the membrane. This latter fact

[1] ROBERTSON, T. B., "The Physical Chemistry of the Proteins," p. 297, New York, London, Bombay, Calcutta, and Madras, 1918.

[2] LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 667, 1920–21.

seems a complete proof for the correctness of Donnan's theory of membrane equilibrium, and also a further proof for the correctness of the purely chemical conception of the combination of proteins with acids and alkalies. For unless the proteins form true ionizable salts with acids and alkalies they cannot fulfill the requirements of the Donnan equilibrium.

It was, however, possible to go a step further, inasmuch as these membrane potentials showed the typical colloidal characteristics noticed in connection with viscosity, swelling, and osmotic pressure, namely, the potential difference across the membrane was depressed by the addition of neutral salts, was increased by the addition of little acid to isoelectric protein, and depressed by the addition of more acid; the depressing effect was in both cases due to the ion with the opposite sign of charge to that of the protein ion, and finally the depressing influence increased rapidly with the valency of the active ion—while the other characteristics of the ion aside from sign and valency had no effect. In this case there was not the slightest doubt that the effects were exclusively the result of the Donnan equilibrium since they could be mathematically predicted and calculated from the equilibrium formula.

The writer was able to show, in addition, that the analogous behavior of the osmotic pressure and viscosity of protein solutions could be explained and calculated on the basis of Donnan's theory.

It, therefore, turns out that two laws of classical chemistry suffice to explain colloidal behavior quantitatively and mathematically, and these two laws are the stoichiometrical law and Donnan's theory of membrane equilibria. The proof for this statement is the purpose of this volume.

CHAPTER II

QUALITATIVE PROOF OF THE CORRECTNESS OF THE CHEMICAL VIEWPOINT

Preparation of Proteins Free from Ionogenic Impurities

The first problem confronting the chemist is to find a method which permits him to settle definitely the problem whether only one or both ions of a salt combine with a protein. This decision was not possible with the old methods. Those who believe in the adsorption theory assume that both ions of a salt are adsorbed by colloids and Pauli holds that both ions of a salt are adsorbed by the non-ionized molecules of protein.[1]

When a block of gelatin is put into a salt solution, the solution enters into the interstices between the gelatin molecules constituting the block. When such a block of gelatin is melted, of course, both ions of the salt are found, but nobody can tell whether the salt found was only the salt contained in the interstices of the original gel or whether it was in combination with the gelatin. This difficulty can be circumvented by using solid gelatin in the form of a very fine powder of grains approximately equal in size. When such powdered gelatin is exposed to a salt solution for some time, we can ascertain with certainty by a process of washing whether one or both ions are in combination with the gelatin. After a small mass of the powdered gelatin has been exposed to a salt solution for about 1 hour, it is put on a filter and perfused, with stirring, about six times or more with 25 c.c. of ice-cold distilled water. The water must be cold since otherwise the granules will coalesce, rendering the process of washing futile. By this procedure it is possible to remove the salt solution between the granules of gelatin, without removing the ions in chemical combination with the gelatin—at least not by the six washings. By using this method of washing we can ascertain

[1] Pauli, W., *Fortschr. naturwiss. Forschung*, vol. 4, p. 223, 1912.

whether both or only one of the two oppositely charged ions of a salt enters into combination with gelatin.

Such experiments show that at a given hydrogen ion concentration either the cation or only the anion or neither ion can combine with a protein; and that it depends solely on the hydrogen ion concentration of the solution which of the three possibilities exists.[1]

Proteins are amphoteric electrolytes which exist in three states, according to their hydrogen ion concentration, namely, (*a*) as non-ionogenic or isoelectric protein; (*b*) metal proteinate (*e.g.*, Na or Ca proteinate); and (*c*) protein-acid salts (*e.g.*, protein chloride, protein sulphate, etc.). We will use gelatin as an illustration. At one definite hydrogen ion concentration, namely, that of the isoelectric point, which in the case of gelatin lies at $10^{-4.7}$N (or in Sørensen's logarithmic symbol at pH = 4.7), gelatin can combine practically with neither anion nor cation of an electrolyte. At a pH > 4.7, gelatin can combine only with cations (forming metal gelatinate, *e.g.*, Na gelatinate); at a pH < 4.7, gelatin combines with anions (forming gelatin chloride, etc.). This was proved in the following way: Doses of 1 gm. of finely powdered commercial gelatin (going through sieve 60 but not through 80), which happened to have a pH of 7.0, were brought to different hydrogen ion concentrations by putting them for 1 hour at about 15°C. into 100 c.c. of HNO_3 solutions varying in concentration from M/8,192 to M/8. Owing to the Donnan equilibrium the hydrogen ion concentration inside a gelatin granule is lower than that outside. After this, each dose of 1 gm. of gelatin was put on a filter, the acid being allowed to drain off, and each dose was washed once or twice with 25 c.c. of cold water (at 5°C. or less) to remove the greater part of the acid between the granules of the powdered gelatin. These different doses of originally 1 gm. of gelatin, each of which now possessed a different pH, were put for 1 hour each into a separate beaker containing the same concentration, *e.g.*, M/64, of silver nitrate at a temperature of 15°C. Each dose of powdered gelatin was then put on a filter and washed with stirring six or eight times each with 25 c.c. of ice-cold water. This washing serves the purpose of removing the $AgNO_3$ held in solution between the granules, thus allowing

[1] Loeb, J., *J. Gen. Physiol.*, vol. 1, pp. 39, 237, 1918–19. *Science*, vol. 52, p. 449, 1920. *J. chim. physique*, vol. 18, p. 283, 1920.

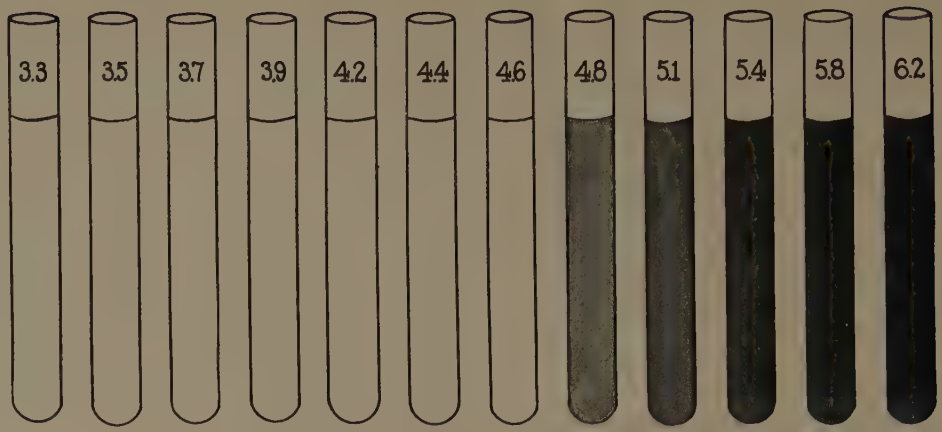

FIG. 1.—Proof that cations combine with proteins only on the alkaline side of the isoelectric point. Powdered gelatin brought to different pH was treated in a dark room with M/64 $AgNO_3$ and then washed with cold water to remove the silver not in combination with gelatin. The gelatin was liquefied, brought to a 1 per cent solution, and the pH was determined. The solutions were then poured into test-tubes and exposed to light. In about half an hour the gelatin of pH >4.7 was dark while the gelatin of pH 4.7 or less remained permanently clear though exposed to light for over a year. The pH of each gelatin solution is marked at the head of each test-tube.

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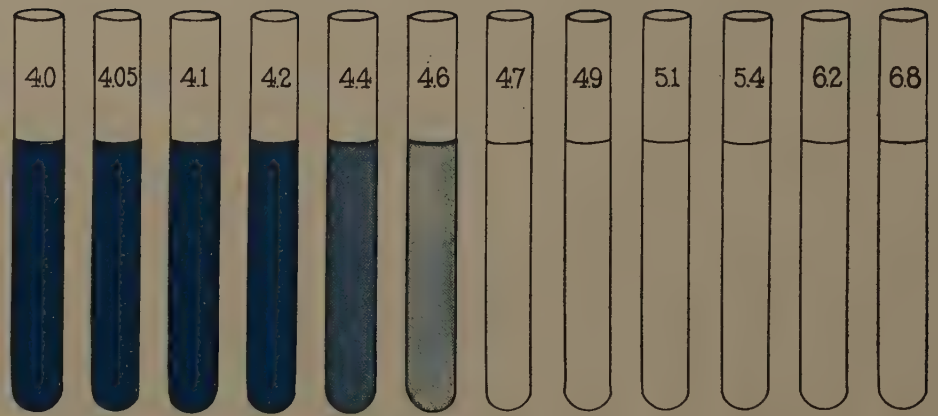

FIG. 2.—Proof that anions combine with proteins only on the acid side of the isoelectric point. Doses of powdered gelatin solutions of different pH were treated with M/128 $K_4Fe(CN)_6$ and then washed with cold water. All the samples of gelatin solution of pH < 4.7 turned blue (through the formation of some ferric salt), while all the gelatin solutions of pH 4.7 or above remained colorless.

us to ascertain where the Ag is in combination with gelatin and where it is not in combination, since the Ag not in combination with gelatin can be removed by the washing while the former cannot, or at least only extremely slowly (by altering the pH). After having removed the $AgNO_3$ not in combination with gelatin by washing with cold water, the gelatin is melted by heating to 40°C., enough distilled water is added to bring the volume of each gelatin solution to 100 c.c., the pH of a sample of each solution is determined potentiometrically, and the solutions are exposed in test-tubes to light, the previous manipulations having been carried out in a dark room (with the exception of the determination of pH, for which only part of the gelatin solution was used). In 20 minutes all the gelatin solutions with a $pH > 4.7$, *i.e.*, from pH 4.8 and above, upon exposure to strong light become opaque and then brown or black, while all the solutions of $pH < 4.7$, *i.e.*, from 4.6 and below, remain transparent even when exposed to light for months or years (Fig. 1). The solutions of $pH = 4.7$ become opaque, but remain white, no matter how long they may have been exposed to light. At this pH—the isoelectric point—gelatin is not in combination with Ag, but it is sparingly soluble. Hence, the cation Ag is only in chemical combination with gelatin when the pH is >4.7. At pH 4.7 or below gelatin is not able to combine with Ag ionogenically. This statement was confirmed by volumetric analysis.

The same tests can be made for any other cation the presence of which can be easily demonstrated. Thus, when powdered gelatin of different pH is treated with $NiCl_2$, and the $NiCl_2$ not in combination with gelatin be removed by washing with cold water, the presence of Ni can be demonstrated in all gelatin solutions with a $pH > 4.7$ by using dimethylglyoxime as an indicator. All gelatin solutions of pH of 4.8 or above assume a crimson color upon the addition of dimethylglyoxime, while all the others remain colorless. If we use copper instead of Ag or Ni as a cation, treating gelatin with copper acetate, and washing afterwards, the gelatin is blue and opaque when its pH is 4.8 or above, but is colorless and clear for $pH < 4.7$. Most striking are the results with basic dyes, *e.g.*, basic fuchsin or neutral red, after sufficient washing with cold water; only those gelatin solutions are red whose pH is above 4.7, while the others are colorless.

On the acid side of the isoelectric point, *i.e.*, at pH<4.7, the gelatin is in combination with the anion of the salt used. This can be demonstrated in the same way by bringing different doses of powdered gelatin to different pH and treating them for 1 hour with a dilute solution of a salt whose anion easily betrays itself, *e.g.*, M/128 $K_4Fe(CN)_6$. If after this treatment the powdered gelatin is washed six times or oftener with cold water to remove the $Fe(CN)_6$ not in chemical combination with gelatin and if 1 per cent solutions of these different samples of gelatin are made, it is found that when the pH is <4.7 the gelatin solution turns blue after a few days (due to the formation of ferric salt), while solutions of gelatin with a pH of 4.7 or above remain permanently colorless (Fig. 2). Hence, gelatin enters into chemical combination with the anion $Fe(CN)_6$ only when pH is <4.7. The same fact can be demonstrated through the addition of ferric salt when gelatin has been treated with NaCNS, the anion CNS being in combination with gelatin only where the pH is <4.7. Acid dyes, like acid fuchsin, combine with gelatin only when the pH is <4.7.[1]

In this way it can be shown that when the pH is >4.7 gelatin can combine only with cations; when the pH is <4.7 gelatin can combine only with anions, while at pH 4.7 (the isoelectric point) gelatin can combine with neither anion nor cation. The idea that both ions are adsorbed or combine with a protein simultaneously is no longer tenable, since otherwise both ions of the salt should have been discovered on both sides of the isoelectric point.

It follows also that a protein solution is not adequately defined by its concentration of protein but that the hydrogen ion concentration must also be known, since each protein occurs in three different forms—possibly isomers—according to its hydrogen ion concentration.

Let us now return once more to the experiment in which doses of powdered gelatin were brought to a different pH and subse-

[1] In these experiments it may happen that a few individual granules do not give off their stain at the isoelectric point or on the alkaline side of the isoelectric point, due probably to experimental shortcomings. When the gelatin is melted the solution may show an indication of red. The difference between the gelatin on the alkaline and on the acid side is, however, sufficiently striking even if this slight error interferes.

quently treated for 1 hour with the same concentration of $AgNO_3$, *e.g.*, M/64 $AgNO_3$, and then washed. In this case, the exposure to light showed us that silver gelatinate existed only on the alkaline side of the isoelectric point, since only on that side did the gelatin turn black. When we now add enough alkali to the gelatin solutions with a pH of 4.6 or less to bring their pH to 4.8 or above, they will *not* turn black when exposed to light. This shows that the gelatin of pH below 4.6 did not contain any demonstrable quantity of silver.[1] It was conceivable that such gelatin of pH below 4.6 contained Ag in a non-ionogenic form. If this were the case, this fact should have betrayed itself in a blackening upon the addition of enough alkali to bring the pH above 4.7.

When we bring powdered gelatin of pH >4.7, which has been treated with M/64 $AgNO_3$ and washed, to a pH of 4.7, or below, the silver which was in combination with the gelatin can be removed by washing with cold water, and such gelatin will not turn black when subsequently exposed to light, provided the washing had been adequate.

When we include that part of the gelatin molecule which cannot react with other electrolytes in brackets, while the part of the molecule which is capable of reacting with other electrolytes is kept outside the brackets, we can symbolize our results in the following way:

Isoelectric gelatin is entirely inside the brackets since at the isoelectric point gelatin can combine neither with anions nor with cations,

$$\left[R\begin{matrix}-NH_2\\-COOH\end{matrix}\right]$$

On the alkaline side from the isoelectric point practically only COOH groups of the molecule are capable of reacting with other compounds and we represent the protein molecule on this side in the following form:

[1] This dogmatic presentation of our results is only approximately correct, since a trace of anion should also combine, theoretically at least, on the alkaline side of the isoelectric point; and a trace of cation on the acid side, at least near the isoelectric point. As a matter of fact, however, this cannot be demonstrated, though the theory of amphoteric electrolytes demands that this should be so.

$$\left[R\begin{matrix}-\overline{NH_2}\\-COOH\end{matrix}\right.$$

Such proteins behave as if they were simple (probably polybasic) fatty acids, the rest of the molecule not participating in the reaction. In the presence of a hydroxide, *e.g.*, NaOH, sodium proteinate is formed

$$\left[R\begin{matrix}-\overline{NH_2}\\-COOH\end{matrix}\right. + NaOH = \left[R\begin{matrix}-\overline{NH_2}\\-COONa\end{matrix}\right. + H_2O$$

and the sodium proteinate dissociates electrolytically into a protein anion and a Na^+ ion

$$\left[R\begin{matrix}-\overline{NH_2}\\-COONa\end{matrix}\right. \rightleftharpoons \left[R\begin{matrix}-\overline{NH_2}\\-\overline{COO}\end{matrix}\right. + Na^+$$

When other electrolytes are present they can of course exchange their cation with the Na of the protein salt. Our symbol considers only one COOH group, but it is probable that as a rule more than one COOH group of a protein molecule combines with alkali (Bugarszky and Liebermann, Sackur, Robertson, Sørensen, Pauli, Northrop[1]).

On the acid side of the isoelectric point only the NH_2 groups of the molecule are capable of reacting with other compounds and we represent the protein molecule on this side in the following form:

$$\left[R\begin{matrix}-NH_2\\-\overline{COOH}\end{matrix}\right.$$

In this form the proteins behave like NH_3 which according to Werner[2] is capable of adding an acid, *e.g.*, HCl, the H ion of the acid being added directly to the N while the Cl remains outside the ring of the 4H in the following way: $\begin{matrix}H\\HNHCl\\H\end{matrix}$. It has been

[1] Northrop, J. H., *J. Gen. Physiol.*, vol. 3, p. 715, 1920–21.

[2] Werner, A., "Neuere Anschauungen auf dem Gebiete der anorganischen Chemie, 3rd ed., Braunschweig, 1913.

shown by W. Kossel[1] and by Langmuir[2] that this idea of Werner is in perfect harmony with the electronic conception of molecular compounds, and we shall give later in this book a direct proof that it holds for proteins. We can, therefore, say that on the acid side of its isoelectric point the protein particle is able to add acid to its NH_2 groups in the following form:

$$\left[R\begin{matrix}-NH_2\\-COOH\end{matrix}\right] + HCl = \left[R\begin{matrix}-NH_2HCl\\-COOH\end{matrix}\right]$$

which dissociates electrolytically into a protein cation and an anion.

$$R\begin{matrix}-NH_3Cl\\-COOH\end{matrix} \rightleftharpoons R\begin{matrix}-\overset{+}{N}H_3\\-COOH\end{matrix} + \overset{-}{Cl}$$

While our symbol indicates only one NH_2 group in the molecule, it is probable that more than one NH_2 or NH group is capable of adding an acid molecule.

The simplification in the general chemistry of proteins implied in these experiments is considerable. We only need to remember that on the alkaline side of its isoelectric point the protein behaves as if it were a fatty acid, only one or more COOH groups existing in a chemically active form; while on the acid side of its isoelectric point we may again disregard the enormous protein molecule and go on the assumption that the protein consists only of one or a number of NH_2 groups, each capable of adding the hydrogen ion of an acid.

It is possible though not proven that the difference in the behavior of the proteins on the two opposite sides of the isoelectric point is accompanied by an intramolecular change in the protein molecule, and that the protein anion in a metal proteinate may be considered an isomer of the protein cation in protein-acid salt. Such a possibility is suggested by the behavior of indicators the electrolytic dissociation of which is accompanied by an intramolecular change.

When we mix a metal gelatinate, *e.g.*, sodium gelatinate, with another salt, *e.g.*, $MgSO_4$, the Na of the metal gelatinate can be

[1] Kossel, W., *Ann. d. Physik*, vol. 49, p. 229, 1916.

[2] Langmuir, I., *J. Am. Chem. Soc.*, vol. 41, p. 868, 1919.

replaced by the Mg of the $MgSO_4$ resulting in the formation of magnesium gelatinate. The SO_4, however, cannot affect the properties of Na gelatinate since it cannot (or can practically not) combine with the gelatin. When, however, we mix gelatin chloride with $MgSO_4$, only the SO_4 can affect the properties of the gelatin salt, since the SO_4 can replace the Cl in the gelatin chloride resulting in the formation of gelatin sulphate. The Mg, however, cannot (or can practically not) enter into combination with gelatin chloride and hence cannot affect its properties.

When we alter the pH of a gelatin-acid salt, *e.g.*, gelatin chloride, by adding alkali, *e.g.*, NaOH, it will cease to be gelatin chloride as soon as the pH is 4.7 because at this pH the Cl will be given off by the gelatin and the latter will be transformed into the chemically inert isoelectric or non-ionogenic gelatin and into NaCl. The isoelectric gelatin can combine practically neither with anions nor with cations. When we add more NaOH so that the pH is >4.7, Na gelatinate will be formed. At no time can metal gelatinate (*e.g.*, Na gelatinate) and gelatin-acid salt (*e.g.*, gelatin chloride) exist simultaneously (except in traces beyond the limits of analytical demonstration). When we have Na gelatinate and add acid, *e.g.*, HCl, the gelatin salt will give off its Na and become isoelectric gelatin as soon as pH = 4.7. This isoelectric gelatin is chemically inert being practically unable to combine with either anion or cation. When we add more HCl, gelatin chloride will be formed.

These experiments show that proteins behave like amphoteric electrolytes, forming definite salts with acids or bases, but that they cannot combine simultaneously with the cation and the anion of a neutral salt. The idea of the existence of adsorption compounds between non-ionized molecules of proteins and molecules of neutral salts is not in harmony with these experiments.

In 1918 the writer[1] published a simple method of preparing ash-free proteins based on the fact that at the isoelectric point proteins can combine neither with anions nor with cations. Hence, if we wish to prepare gelatin or casein free from ionogenic impurities, we must bring these proteins in powdered form to the isoelectric point and then wash them. This is of importance for all industries using proteins as well as for scientific work. In the

[1] LOEB, J., *J. Gen. Physiol.*, vol. 1, p. 237, 1918–19.

writer's work isoelectric protein was always used as the starting point for experiments.

The procedure for preparing isoelectric protein is simple enough. It is only necessary to determine the pH of a given protein solution potentiometrically, and then to add very gradually as much acid or alkali as is required to bring it to the isoelectric point.

The following method was used to prepare larger quantities of approximately isoelectric gelatin: 50 gm. of commercial powdered Cooper's gelatin, which happened to have a pH of 6.0 to 7.0, were put into 3,000 c.c. of M/128 acetic acid in a jar at 10°C., and stirred frequently. After 30 minutes the supernatant liquid was decanted and fresh M/128 acetic acid at 10°C. was added to equal the original volume. The mass was frequently stirred, and after 30 minutes the acid was again decanted and replaced by an equal volume of distilled water at 5°C. The gelatin was well stirred and then filtered by suction through towel cloth in a Buchner funnel. It was then washed in the funnel five times each with 1,000 c.c. of H_2O at 5°C. After all the water was drained off, the gelatin was transferred from the Buchner funnel into a large beaker which was then heated in a water bath to about 50°C. till the gelatin was melted. The concentration of the gelatin was determined by evaporating to dryness, using 10 c.c. of the melted gelatin in an electric oven at 90 to 100°C. for 24 hours.

One hundred cubic centimeters of a 1 per cent gelatin solution prepared in this way had no more than 1 mgm. of ash—apparently $Ca_3(PO_4)_2$, *i.e.*, the salt contained in the solution was M/30,000. Salt in this concentration does not affect the physical properties of proteins, such as osmotic pressure, viscosity, P.D., swelling or precipitability as will be shown in this volume. The following is a result of an ash determination made by Dr. D. I. Hitchcock on a sample of gelatin selected at random. The stock solution contained 12.69 per cent gelatin.

	Sample No. 1	Sample No. 2
Volume of solution	20 c.c.	10 c.c.
Weight of dry gelatin	2.535 gm.	1.269 gm.
Weight of ash	0.0024 gm.	0.0012 gm.

Obtained qualitative tests for Fe^{+++}, Ca^{++}, and $PO_4^{=}$, negative tests for Cl^- and $SO_4^{=}$.

Miss Field[1] has shown that by carrying the washing process a step further the last traces of ash can be removed from the powdered gelatin. In bringing powdered gelatin to the isoelectric point and washing with water of the pH of the isoelectric point we can quickly make the gelatin completely ash-free. If the protein is soluble at this point (as is the case with crystalline egg albumin) it is only necessary to carry out the dialysis at the pH of the isoelectric point to obtain the protein free from ionogenic impurities.[2]

This fact is a further support of our contention that at the isoelectric point proteins can combine with neither anion nor cation.

We may call attention to one interesting fact which is in harmony with these results. It has always been known that pepsin digestion occurs in nature in an acid medium. The reason for this connection of an acid reaction with pepsin digestion was cleared up by Northrop[3] who found that the hydrogen ion concentration at which pepsin commences to act on a protein varies with the isoelectric point of the protein and that the action always occurs on the acid side of the isoelectric point. It seems to follow from the experiments of Pekelharing and Ringer[4] that pepsin is an anion like Cl which can only combine with a positive protein ion. This combination between pepsin and positive protein ion seems to be the prerequisite for the falling apart (or digestion) of the protein ion.

[1] Field, A. M., *J. Am. Chem. Soc.*, vol. 43, p. 667, 1921.

[2] Miss Field's paper as well as the writer's paper referred to were overlooked by C. R. Smith (*J. Am. Chem. Soc.*, vol. 43, p. 1350, 1921) who also describes a method of preparing ash-free gelatin.

[3] Northrop, J. H., *J. Gen. Physiol.*, vol. 3, p. 211, 1920–21.

[4] Pekelharing, C. A. and Ringer, W. E., *Z. physiol. Chem.*, vol. 75, p. 282, 1911.

CHAPTER III

METHODS OF DETERMINING THE ISOELECTRIC POINT OF PROTEIN SOLUTIONS

The results of the preceding chapter make it clear that whenever work with amphoteric electrolytes is contemplated it becomes necessary to ascertain first the isoelectric point of the substance, since at the isoelectric point the material can be most easily freed from ionogenic impurities. There can be no doubt that many of the substances exhibiting colloidal behavior are amphoteric electrolytes.

Hardy and Michaelis determined the isoelectric point by observations on the migration of particles in the electrical field. There are other methods available for this purpose, some of which are often more convenient than Hardy's original method. These methods are based on the fact that at the isoelectric point the osmotic pressure, the viscosity, the amount of alcohol required for precipitation, the conductivity, the swelling, the P.D. are all a minimum. When the curves representing the values of these properties are plotted as ordinates over the pH as abscissæ, the curves show a sharp drop at the isoelectric point. If, therefore, a protein is brought to different pH by adding acid or alkali, and if any of the properties mentioned is determined, the approximate position of the isoelectric point can be inferred from the minimum point of the property which is used as a test. The writer has found it most convenient to use osmotic pressure experiments in the case of proteins.

The following older experiment by the writer may serve as an illustration.[1] A number of doses each containing 1 gm. of finely powdered Cooper's gelatin which had a pH of a little over 7.0 and consisted partly of Ca gelatinate were put for 30 minutes at 15°C. into beakers containing 100 c.c. of HBr of different concentrations, varying from M/8 to M/8,192; and as a control 1 gm. of gelatin was put for 30 minutes at 15°C. into 100 c.c. of distilled

[1] Loeb, J., *J. Gen. Physiol.*, vol. 1, p. 363, 1918–19.

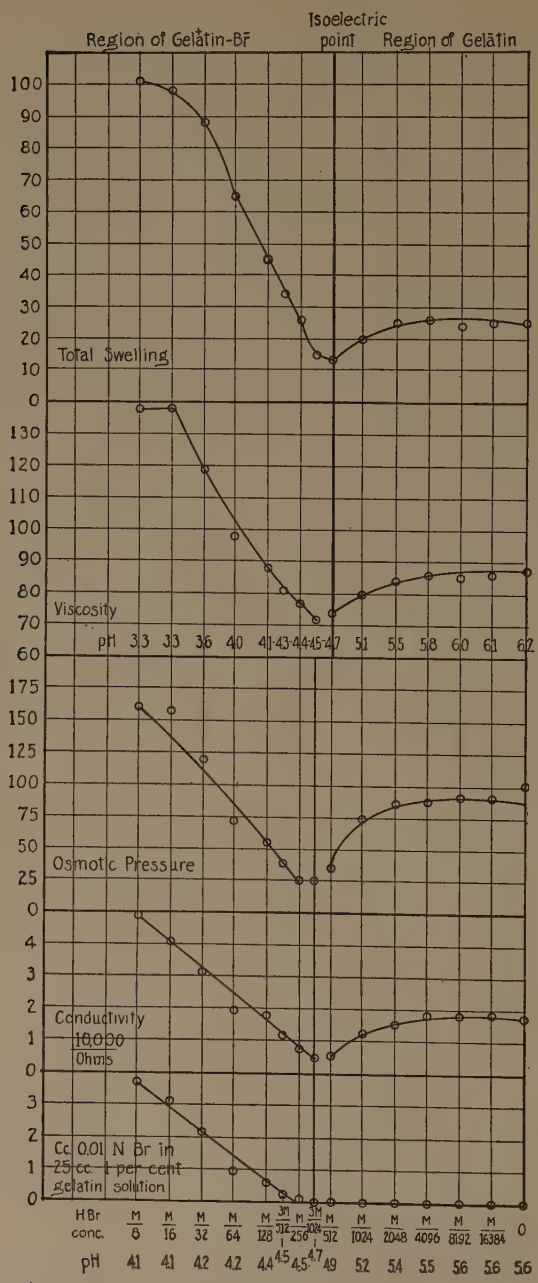
Isoelectric
Region of Gelatin-Br
point
Region of Gelatin
Total Swelling
Viscosity
pH 3.3 3.3 3.6 4.0 4.1 4.3 4.4 4.5 4.7 5.1 5.5 5.8 6.0 6.1 6.2
Osmotic Pressure
Conductivity
10,000
Ohms
Cc 0.01 N Br in
25 cc. 1 per cent
gelatin solution
HBr conc.
M/0 M/16 M/32 M/64 M/128 M/192 M/256 M/384 M/512 M/1024 M/2048 M/4096 M/8192 M/16384 0
pH 4.1 4.1 4.2 4.2 4.4 4.5 4.5 4.7 4.9 5.2 5.4 5.5 5.6 5.6 5.6

FIG. 3.—Showing that the physical properties of gelatin are a minimum at the isoelectric point.

water. The powdered gelatin was then put into a cylindrical funnel and the acid allowed to drain off. The powdered gelatin in the funnel was then perfused six or eight times, with constant stirring, each time with 25 c.c. cold water—*i.e.*, water not above 5°C.—to remove the excess of acid and the salts. The water must be cold to prevent the powdered granules from coalescing since otherwise the washing would be incomplete. After the liquid was drained off from the filter, the volume (*i.e.*, the relative swelling of the gelatin) was measured; then the gelatin was melted by heating to 45°C. and enough water was added to bring the volume in each case to 100 c.c. Then the conductivity, osmotic pressure, and viscosity were measured in a way to be described in a later chapter, and the pH was also determined, either colorimetrically (which gives fairly accurate results with gelatin but not with the other proteins) or preferably with the hydrogen electrode. In the experiment represented in Fig. 3 the pH was measured colorimetrically. A glance at the figure shows that the ordinates of the curves representing the values for osmotic pressure, conductivity, swelling, etc., drop very sharply at pH 4.7, *i.e.*, the isoelectric point of gelatin. By this method the approximate location of the isoelectric point can be recognized at a glance from the osmotic pressure measurements, the conductivity measurements, etc. The P.D. measurements would also show a minimum at the isoelectric point.

The lowest curve in Fig. 3 represents titration for Br. Gelatin should exist in the form of gelatin bromide only on the acid side of the isoelectric point and titration for Br should be negative when the pH is above 4.7. The curve shows that no Br was found when pH was equal or greater than 4.7; while it was found on the acid side increasing in quantity the lower the pH. On the alkaline side of the isoelectric point the gelatin existed still in the state of Ca gelatinate. In this experiment the mass of the gelatin was diminished by solution and washing to 0.8 gm. or possibly a little less.

We shall see later, that when powdered gelatin is put into an acid solution, *e.g.*, N/100 or N/1,000 HBr, the concentration of the acid inside the gelatin granules is considerably lower than in the outside solution. This is due to the establishment of a Donnan equilibrium.

CHAPTER IV

QUANTITATIVE PROOF OF THE CORRECTNESS OF THE CHEMICAL VIEWPOINT

1. The qualitative experiments of the second chapter did not permit us to decide whether ions combine with proteins stoichiometrically (*i.e.*, by the purely chemical forces of primary valency), or according to the empirical rule of adsorption, as is assumed in colloid chemistry. A decision can be rendered by titration experiments.[1]

The titrations required for this proof differ from those usually performed in chemistry. In the usual chemical work titration is carried to the point of neutrality, *i.e.*, pH near 7.0. Proteins, however, are amphoteric electrolytes, the isoelectric point of which is generally different from neutrality. Gelatin and casein act as bases for a pH below 4.7, and if we wish to ascertain how much of a certain acid 1 gm. of isoelectric gelatin can bind we have to titrate to a pH below 4.7. In doing this, we must also remember that at such a high hydrogen ion concentration only strong dibasic acids, like H_2SO_4, continue to dissociate both H ions, while weaker dibasic or tribasic acids, *e.g.*, H_3PO_4, are only able to split off one H ion, acting therefore like monobasic acids.

Our solutions contain generally 1 gm. of isoelectric protein in 100 c.c., and such solutions will be called 1 per cent protein solutions. When 1 per cent solutions of albumin sulphate or 1 per cent solutions of gelatin chloride are mentioned, this means that 1 gm. of originally isoelectric albumin or gelatin was in 100 c.c. of the solution. The concentration of the stock solution of isoelectric gelatin, albumin, or casein was determined by measuring the dry weight of the solution.

When different quantities of 0.1 N acid, *e.g.*, HCl, are added to the same quantity of protein, *e.g.*, 1 gm. of isoelectric gelatin or crystalline egg albumin, bringing the volume of the solution

[1] Loeb, J., *J. Gen. Physiol.*, vol. 1, p. 559, 1918–19; vol. 3, p. 85, 1920–21.

always to 100 c.c., it is found that the resulting hydrogen ion concentration of the solution is different from the pH which is found when the same amount of acid is added to the same quantity of pure water. This is due to the fact that part of the acid combines with the protein as originally suggested by Bugarszky and Liebermann.[1] On the basis of Werner's[2] idea the HCl should combine with the NH_2 groups of the protein molecule in the same way as if it were added to NH_3, thus forming a salt of the type RNH_3Cl. This is intelligible on the basis of the recent theories of G. N. Lewis,[3] Kossel,[4] and Langmuir.[5] Gelatin chloride may therefore be expected to dissociate electrolytically in the following way:

$$\text{Gelatin } NH_3Cl \rightleftharpoons \text{gelatin } \overset{+}{N}H_3 + \overset{-}{Cl}$$

Hence, the concentration of the free Cl ions in a watery solution of HCl should remain the same if a small amount of isoelectric gelatin is added, provided the electrolytic dissociation is complete. This was tested by comparing the pCl of HCl solutions with and without gelatin (Table I). Both the pH and the pCl were measured electrometrically. Table I shows that this pCl was in solutions of HCl without gelatin always identical with the pH of the same solution. In a second set of experiments the same HCl solutions contained each 1 gm. of isoelectric gelatin in 100 c.c., and the pH and pCl in these 1 per cent solutions of gelatin chloride were also determined after the reaction was complete. The reader will notice from Table I that the values for pCl of the watery solutions are within the limits of accuracy of the determinations identical with those found in the gelatin solutions containing the same amount of acid. The pH, however, is different in the aqueous and in the 1 per cent gelatin solutions,

[1] Bugarszky, S. and Liebermann, L., *Arch. ges. Physiol.*, vol. 72, p. 51, 1898.

[2] Werner, A., "Neuere Anschauungen auf dem Gebiete der anorganischen Chemie," 3rd ed., Braunschweig, 1913.

[3] Lewis, G. N., *J. Am. Chem. Soc.*, vol. 38, p. 762, 1916.

[4] Kossel, W., *Ann. Physik*, vol. 49, p. 229, 1916.

[5] Langmuir, I., *J. Am. Chem. Soc.*, vol. 41, p. 868, 1919; vol. 42, p. 274, 1920.

since, in the latter, part of the H ions of the free HCl added becomes part of the complex gelatin cation, gelatin-NH_3^+. The figures in Table I then prove that a strong acid, like HCl, combines with the protein according to Werner's ideas.

TABLE I

Cubic centimeters of 0.1 N HCl in 100 c.c. solution	Solution containing no gelatin		Solution containing 1 gm. of isoelectric gelatin in 100 c.c.	
	pH	pCl	pH	pCl
2	2.72	2.72	4.2	2.68
3	2.52	2.54	4.0	2.53
4	2.41	2.39		
5	2.31	2.29	3.60	2.33
6	2.24	2.26	3.41	2.25
7	2.16	2.18	3.23	2.18
8	2.11	2.12	3.07	2.11
10	2.01	2.01	2.78	2.025
15	1.85	1.85	2.30	1.845
20	1.72	1.76	2.06	1.76
30	1.55	1.59	1.78	1.60
40	1.43	1.47	1.61	1.47

It was found that whenever the same amount of acid was added to the same amount, *e.g.*, 1 gm., of originally isoelectric gelatin, making up the volume to 100 c.c., the pH of the solution was always the same; so that we can say how much Cl is in combination with the protein if we know the pH of the gelatin chloride solution. The lower the pH, the more chloride enters into combination with the protein, until finally, all the protein is transformed into protein chloride.

It seems that when an acid, *e.g.*, HCl, is added to isoelectric gelatin (or any other isoelectric protein), an equilibrium is established between free HCl, protein chloride, and non-ionogenic (or isoelectric) protein; when alkali is added to isoelectric gelatin, an equilibrium is established between metal proteinate, non-ionized protein, and free alkali (above pH 4.7). Similar results had been obtained by Sørensen.[1]

[1] SØRENSEN, S. P. L., Studies on proteins: *Compt. rend. trav. Lab. Carlsberg*, vol. 12, Copenhagen, 1915–17.

It can be shown by titration experiments that acids and bases combine with proteins in the same way as they combine with crystalline compounds, namely, by the purely chemical forces of primary valency. It is known that a weak dibasic or tribasic acid gives off one hydrogen ion more readily than both or all three, and that it depends on the hydrogen ion concentration of the solution whether one or two or three H ions are dissociated from a tribasic acid. Thus H_3PO_4 will give off only one H ion as long as the pH is below 4.6. Oxalic acid, which is a stronger acid, will act like a monobasic acid below a pH of about 3.0,[1] while above this pH it acts more and more like a dibasic acid. In a strong dibasic acid, like H_2SO_4, both H ions are held with so small an electrostatic force that even at a pH of 3.0 or considerably below the acid acts as a dibasic acid. If the forces which determine the reaction between these acids and proteins are purely chemical, it would follow that three times as many cubic centimeters of 0.1 N H_3PO_4 should be required to bring 100 c.c. of 1 per cent solution of isoelectric gelatin to a given pH below 4.6, *e.g.*, 3.0, as are required in the case of HNO_3 or HCl; while it should require just as many cubic centimeters of 0.1 N H_2SO_4 as of 0.1 N HCl. Twice as many cubic centimeters of 0.1 N oxalic acid should be required to bring isoelectric gelatin to a pH of 3.0 or below, as are required in the case of HCl. It can be shown that these predictions are true.[2]

2. Crystalline egg albumin was prepared according to Sørensen's method,[3] and crystallized three times. The only difference in procedure was in the dialysis. Instead of putting the water under negative pressure, as was done by Sørensen, pressure was put on the egg albumin by attaching a long glass tube full of water to the dialyzing bag so that the solution was under about 150 cm. water pressure during dialysis. This was necessary to avoid too great an increase in volume. The same stock solution

[1] Hildebrand, J. H., *J. Am. Chem. Soc.*, vol. 35, p. 847, 1913. See also Michaelis, L., "Die Wasserstoffionenkonzentration," Berlin, 1914; Clark, W. M., "The Determination of Hydrogen Ions," Baltimore, 1920.

[2] The experiments to be described are from Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920–21.

[3] Sørensen, S. P. L., Studies on proteins: *Compt. rend. trav. Lab. Carlsberg*, vol. 12, Copenhagen, 1915–17.

of albumin served for all the experiments and was diluted before the experiment to a 1 per cent solution. The concentration of

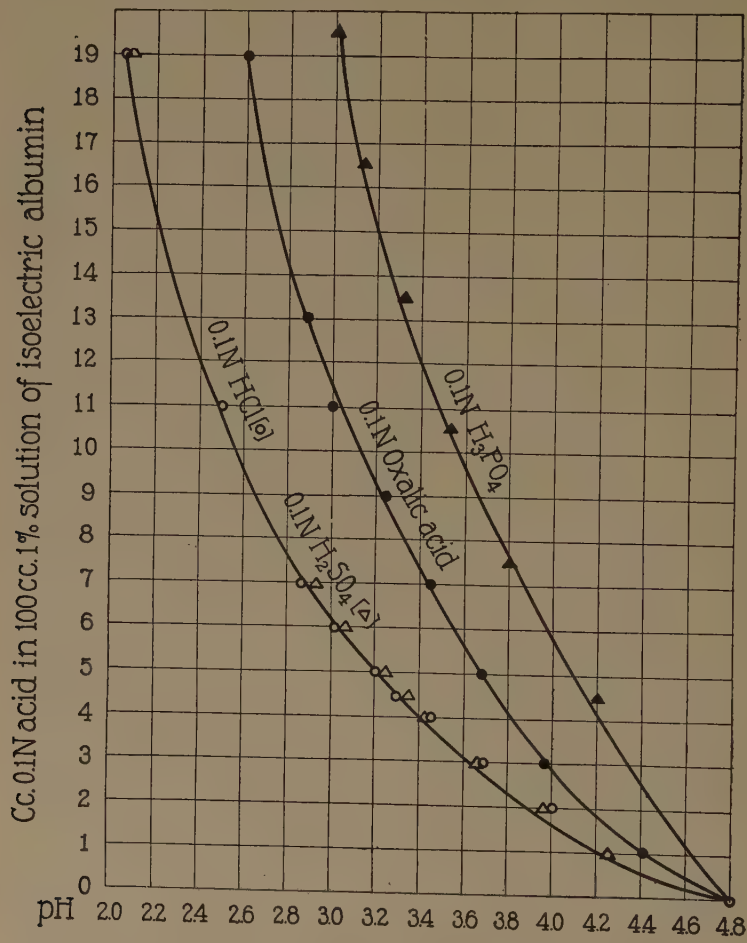

FIG. 4.—The ordinates represent the number of c.c. of 0.1 N HCl, H_2SO_4, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric crystalline egg albumin to the pH indicated on the axis of abscissæ. Enough H_2O was added to bring the albumin and acid to a volume of 100 c.c. For the same pH the ordinates for HCl, H_2SO_4, and phosphoric acid are approximately as 1:1:3. The ratio of HCl to oxalic acid is a little less than 1:2 when pH is > 3.0.

ammonium sulphate left in the solution was between M/1,000 and M/2,000. The pH of the stock solution was about 5.20. By

adding about 1 c.c. 0.1 N HCl to 100 c.c. of a 1 per cent solution of this albumin the solution was brought to the isoelectric point of the egg albumin, which is according to Sørensen at pH = 4.8.

The 1 per cent solutions were made up with different quantities of acid (or alkali) and the pH of the albumin solution was determined electrometrically. In Fig. 4 are plotted the titration curves in which the pH are the abscissæ while the ordinates are the cubic centimeters of 0.1 N acid required to bring the 1 per cent solutions of originally isoelectric crystalline egg albumin to different pH. The curves represent these titration values for four acids, HCl, H_2SO_4, H_3PO_4, and oxalic acid. Beginning with the lowest curve, we notice that the curve is the same for 0.1 N HCl and 0.1 N H_2SO_4, since both are strong acids; or, in other words, H_2SO_4 combines in equivalent proportions with egg albumin. The curve for H_3PO_4 is the highest curve and if we compare the values for H_3PO_4 with those for HCl (or H_2SO_4) we notice that for each pH the ordinate for H_3PO_4 is as nearly three times as high as that for HCl as the accuracy of our experiments permits. This means that phosphoric acid combines with albumin (inside of the range of pH of our experiment) in molecular proportions and that the anion of albumin phosphate is the monovalent anion H_2PO_4.

The values for oxalic acid are for pH below 3.2 almost but not quite twice as high as those for HCl, indicating that for these values of pH oxalic acid combines to a greater extent in molecular and only to a small extent in equivalent proportions with albumin.

These combining ratios of the four acids named with crystalline egg albumin are, therefore, the same as those which would be found if we substituted the crystalloidal base NH_3 for the colloid egg albumin, titrating in the same range of pH.

From the curves just discussed, the amount of acid in combination with 1 gm. of originally isoelectric crystalline egg albumin in a 1 per cent solution of this protein at different pH can easily be calculated. Let us assume the acid added to isoelectric albumin to be HCl. If, *e.g.*, at pH 3.0, 6 c.c. of 0.1 N HCl are contained in 100 c.c. of the 1 per cent solution of the originally isoelectric albumin (as indicated in Fig. 4), part of the acid is in combination with the albumin and part is free. How much is

free is known from the pH of the albumin chloride solution, namely, 1 c.c., since in the example selected the pH is 3.0 (Fig. 4). If 1 c.c. is deducted from 6 c.c. it is found that at pH 3.0

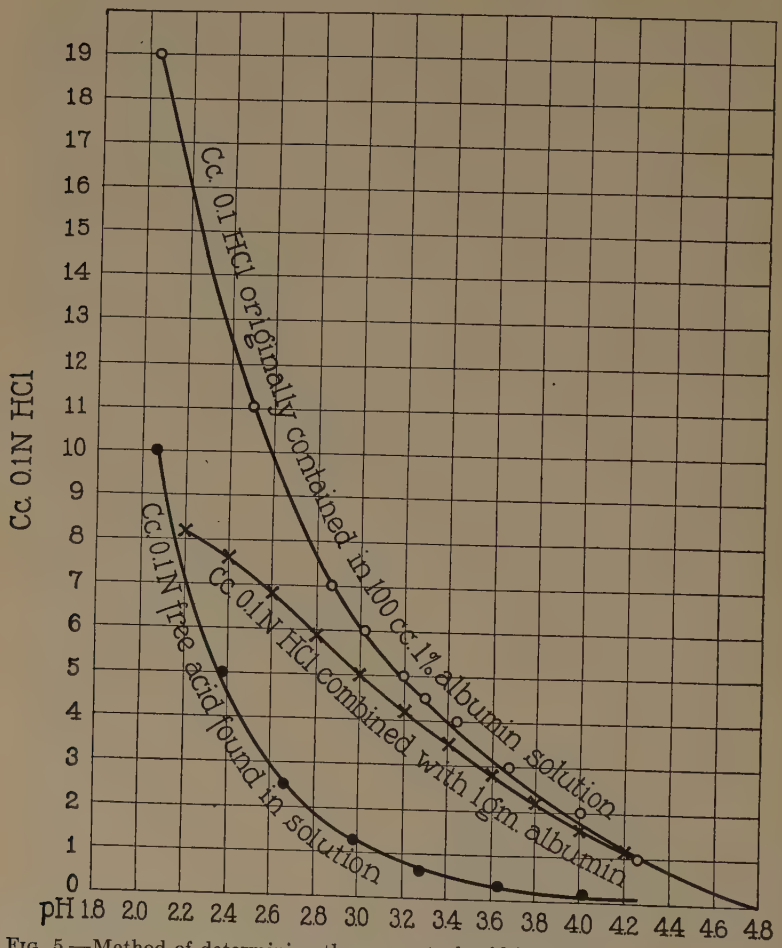

FIG. 5.—Method of determining the amount of acid in combination with 1 gm. of albumin from titration curve and pH curve.

5 c.c. of 0.1 N HCl are in combination with 1 gm. of originally isoelectric crystalline egg albumin in 100 c.c. solution (Fig. 5). A curve is constructed in which the abscissæ are the pH while the ordinates are the cubic centimeters of 0.1 N HCl contained in 100

c.c. of a watery solution, *without protein*. If the ordinates of this

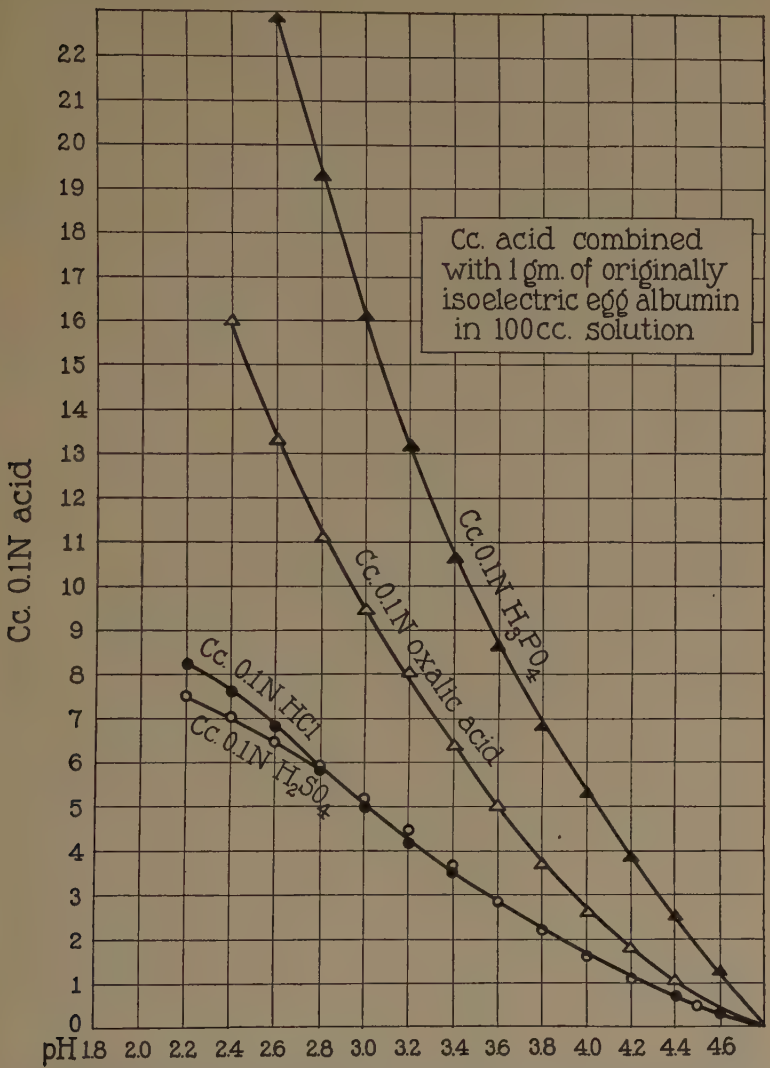
Cc. acid combined with 1 gm. of originally isoelectric egg albumin in 100 cc. solution
Cc. 0.1N acid
pH
cc. 0.1N H_3PO_4
cc. 0.1N oxalic acid
cc. 0.1N HCl
cc. 0.1N H_2SO_4

FIG. 6.—Proof of the stoichiometrical character of the combination of acids with isoelectric albumin. The same mass of albumin combines with three times as many c.c. of 0.1 N H_3PO_4 as with HCl or H_2SO_4; and with twice as many c.c. of 0.1 N oxalic acid below pH 3.0.

latter curve are deducted from the ordinates of the titration

curve in Fig. 4 containing 1 per cent of originally isoelectric albumin chloride we get a curve whose ordinates give the number of cubic centimeters of 0.1 N HCl in actual combination with 1 gm. of originally isoelectric albumin in 100 c.c. solution (middle curve Fig. 5).

Figure 6 contains the curves whose ordinates give the amount of cubic centimeters of 0.1 N HCl, H_2SO_4, $H_2C_2O_4$, and H_3PO_4 in combination with 1 gm. of originally isoelectric egg albumin at different pH. It appears again that the curves for HCl and H_2SO_4 practically coincide as the purely chemical theory demands, that the oxalic acid curve is higher, and that the phosphoric acid curve is still higher. What is of greater importance is that for the same pH the ordinates of the H_3PO_4 curve are always approximately three times as high as the ordinates of the curves for HCl and H_2SO_4.

The results in Table II show the actual numbers of cubic centimeters of each of the four acids in combination with 1 gm. of originally isoelectric crystalline egg albumin in 100 c.c. solution. The values for HCl and H_2SO_4 are identical. Those for H_3PO_4 are within the limits of accuracy always three times as large as those for HCl. Thus at pH 4.0, 1.7 c.c. of 0.1 N HCl or H_2SO_4 are combined with 1 gm. of albumin, while 5.3 c.c. of 0.1 N H_3PO_4 are in combination; at 3.4, 3.5 c.c. of 0.1 N HCl or H_2SO_4 and 10.6 c.c. of 0.1 N H_3PO_4.

In the case of oxalic acid, we notice that at pH above 3.6 the number of cubic centimeters of 0.1 N oxalic acid in combination with 1 gm. of albumin is less than twice that of HCl and that the difference is the greater the higher the pH. At pH = 3.2 and below practically twice as many cubic centimeters of oxalic acid are at the same pH in combination with 1 gm. of originally isoelectric albumin as are of HCl. Thus at pH 2.6, 6.7 c.c. of 0.1 N HCl and 13.3 c.c. of 0.1 N oxalic acid are in combination with 1 gm. of albumin; at pH 3.0, 5.0 c.c. of 0.1 N HCl and 9.5 c.c. of 0.1 N oxalic acid. These figures correspond to the results to be expected on the basis of Hildebrand's titration experiments against inorganic bases. These titration experiments then leave no doubt that these acids combine with proteins in the same stoichiometric way as they combine with crystalloids. That these simple facts had not been discovered earlier is the con-

TABLE II.—CUBIC CENTIMETERS OF 0.1 N ACID IN COMBINATION WITH 1 GM. OF ORIGINALLY ISOELECTRIC CRYSTALLINE EGG ALBUMIN IN 100 C.C. SOLUTION

pH	HCl, cubic centimeters	H_2SO_4, cubic centimeters	Oxalic acid, cubic centimeters	H_3PO_4, cubic centimeters
4.2	1.15	1.15	1.8	3.8
4.0	1.7	1.7	2.6	5.3
3.8	2.3	2.3	3.7	6.8
3.6	2.9	2.9	5.0	8.6
3.4	3.5	3.5	6.3	10.6
3.2	4.2	4.3	8.0	13.1
3.0	5.0	5.1	9.5	16.1
2.8	5.8	5.9	11.1	19.3
2.6	6.7	6.5	13.3	22.9
2.4	7.6	7.0	16.0	

sequence of the failure of the workers to consider the hydrogen ion concentration of their solutions. Had this been done, nobody would have thought of suggesting that acids combine with proteins according to the adsorption formula.

These titration experiments are of especial value for the reason that crystalline egg albumin is for the present probably the purest protein available.

The same proof can be furnished in the case of other proteins, *e.g.*, gelatin. A stock solution of isoelectric gelatin was used for the experiment. The isoelectric gelatin was prepared by putting the powdered gelatin of pH 7.0 into M/128 acetic acid (100 c.c. of M/128 acid for 1 gm. of gelatin) for 1 hour at 15°C., and then washing four or five times with cold water (5°C.). An 8 per cent stock solution was prepared; the concentration of the gelatin was ascertained by a determination of the dry weight. To 50 c.c. of a 2 per cent solution of isoelectric gelatin were added different quantities of acid and the volume made up to 100 c.c. by adding enough H_2O, usually of a pH of about 5.6. It was ascertained how many cubic centimeters of 0.1 N different acids were required to bring 1 gm. of isoelectric gelatin in a 1 per cent solution to the same pH.

In Fig. 7 the abscissæ are the pH while the ordinates are the number of cubic centimeters of 0.1 N HCl, H_2SO_4, H_2 oxalate,

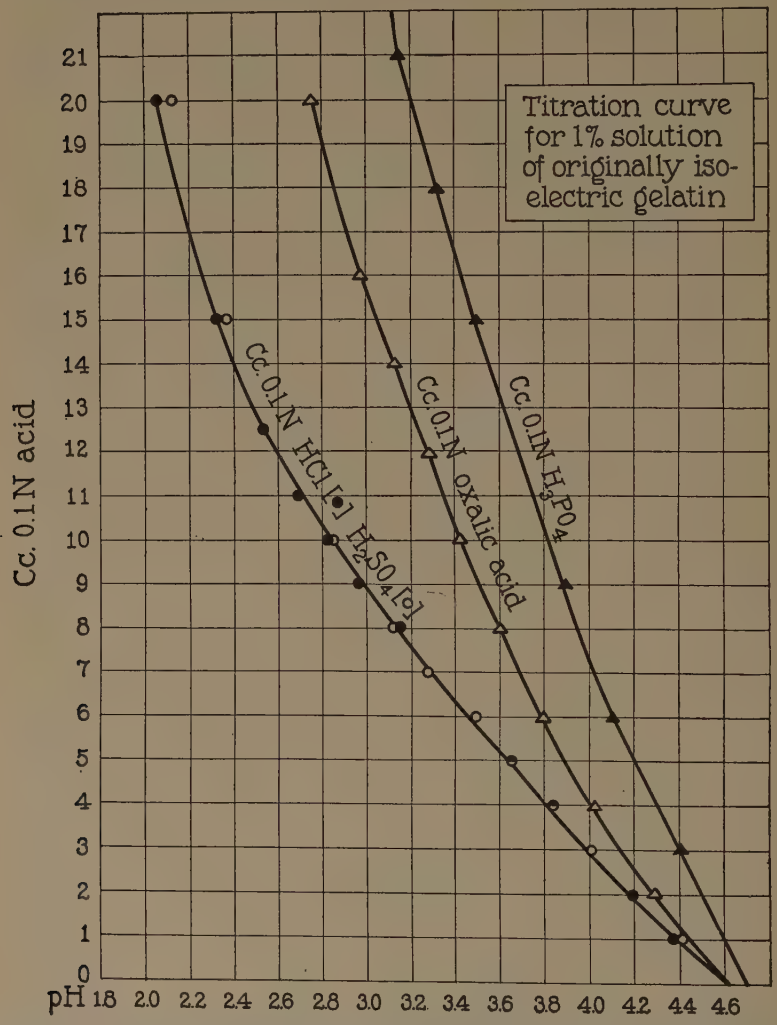
Titration curve for 1% solution of originally iso-electric gelatin
Cc. 0.1 N acid
pH
Cc. 0.1N HCl [7]
Cc. 0.1N H_2SO_4 [7]
Cc. 0.1N oxalic acid
Cc. 0.1N H_3PO_4

FIG. 7.—Titration curve for 1 per cent solution of originally isoelectric gelatin, proving the stoichiometrical character of combination of acids with gelatin (see legend under Fig. 6).

and H_3PO_4 contained in 100 c.c. solution of originally isoelectric gelatin to the same pH.

It is again obvious that the curves for HCl and H_2SO_4 are practically identical while the ordinates of the curve for H_3PO_4

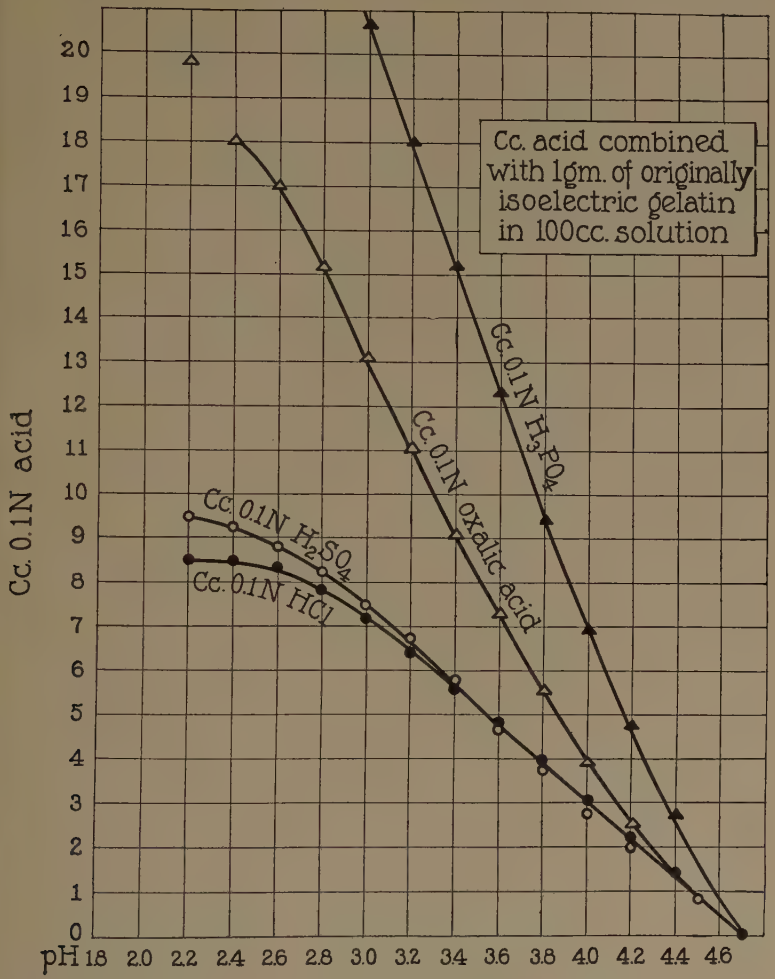
Cc. acid combined with 1gm. of originally isoelectric gelatin in 100cc. solution
Cc. 0.1N acid
pH
Cc. 0.1N H_3PO_4
Cc. 0.1N oxalic acid
Cc. 0.1N H_2SO_4
Cc. 0.1N HCl
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6

FIG. 8.—Combination curve of acids with gelatin, confirming the stoichiometrical character of the combination.

are approximately three and those for oxalic acid about twice as high as those for HCl or H_2SO_4 for the same pH, as long as the pH is below 3.2; while above 3.2 the curve for oxalic acid deviates

the more from that ratio the higher the pH, as the theory demands.

The curves in Fig. 8 represent the values for the cubic centimeters of 0.1 N acid found in combination with 1 gm. of originally isoelectric gelatin in 100 c.c. solution at different pH. The results are tabulated in Table III. The table shows that within the limits of accuracy of the experiments, at the same pH approximately equal numbers of cubic centimeters of 0.1 N HCl and 0.1 N H_2SO_4 are in combination with 1 gm. of originally isoelectric gelatin in 100 c.c. solution, while about three times as many cubic centimeters of 0.1 N H_3PO_4 are in combination. The number of cubic centimeters of 0.1 N oxalic acid in combination with 1 gm. of gelatin is less than twice that of HCl as long as the pH is above 3.0, while below 3.0 the combining ratio of the two acids is approximately as 1:2, as the theory demands.

TABLE III.—CUBIC CENTIMETERS OF 0.1 N ACID IN COMBINATION WITH 1 GM. OF ORIGINALLY ISOELECTRIC GELATIN IN 100 C.C. SOLUTION

pH	HCl, cubic centimeters	H_2SO_4, cubic centimeters	Oxalic acid, cubic centimeters	H_3PO_4, cubic centimeters
4.0	....	2.7	3.9	6.95
3.8	3.9	3.75	5.5	9.4
3.6	4.8	4.8	7.3	12.3
3.4	5.6	5.75	9.1	15.2
3.2	6.4	6.75	11.0	18.0
3.0	7.2	7.5	13.15	20.7
2.8	7.9	8.25	15.3	23.6
2.6	8.35	8.8	17.1	26.2
2.4	8.5	9.3	18.0	....

These experiments corroborate our conclusion that acids combine stoichiometrically with proteins if the hydrogen ion concentration is properly taken into consideration.

Similar experiments were made with casein prepared after the method of L. L. Van Slyke and J. C. Baker,[1] who described in

[1] VAN SLYKE, L. L. and BAKER, J. C., *J. Biol. Chem.*, vol. 35, p. 127, 1918.

1918 a method for preparing "pure casein" from skimmed milk, which consisted

"in the gradual addition of acid and its immediate distribution through the mass of milk without causing coagulation of casein at the point where the acid first comes into contact with a portion of the milk. This result can be accomplished by introducing the acid below the surface of the milk with high-speed mechanical stirring. After standing under gentle stirring for 3 hours with acidity just below the point of casein coagulation, addition of acid is continued slowly, accompanied as before by rapid stirring in order to obtain the particles of casein coagulum in the finest possible state of division."

The coagulated casein is then centrifuged and after repeated washings is found free from Ca and inorganic P. As Van Slyke and Baker point out, the pH of this casein coagulum is about 4.5 to 4.6, *i.e.*, it is slightly below the isoelectric point. The essential feature of Van Slyke and Baker's method therefore consists in slowly bringing the milk or casein solution approximately to the pH of the isoelectric point of casein. The writer has shown that gelatin gives off all ionogenic impurities at the isoelectric point and Van Slyke and Baker's experiments show that the same method works also with casein. The casein prepared after Van Slyke and Baker's method is also free from albumin since this latter protein is soluble at pH 4.5 or 4.7, and is, hence, removed from the insoluble isoelectric casein by washing.

In our experiments[1] we used casein prepared after Van Slyke and Baker's method from skimmed milk and in addition from a "pure casein" of the market. Both preparations gave practically the same result. In order to remove traces of fat from the casein the latter was washed in acetone.

It is not possible to prepare 1 per cent casein solutions, except with a few acids, on account of the low solubility of the casein salts with acids. It is, however, possible to compare casein chloride and casein phosphate in 1 per cent solutions. One gram of isoelectric casein, prepared after Van Slyke and Baker, was put into 100 c.c. of watery solution containing 1, 2, 3, etc., c.c. of 0.1 N HCl or 0.1 N H_3PO_4. The pH of the casein solution was

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, 547, 1920–21.

ascertained potentiometrically and the number of cubic centimeters of 0.1 N acid required to bring the 1 per cent casein solu-

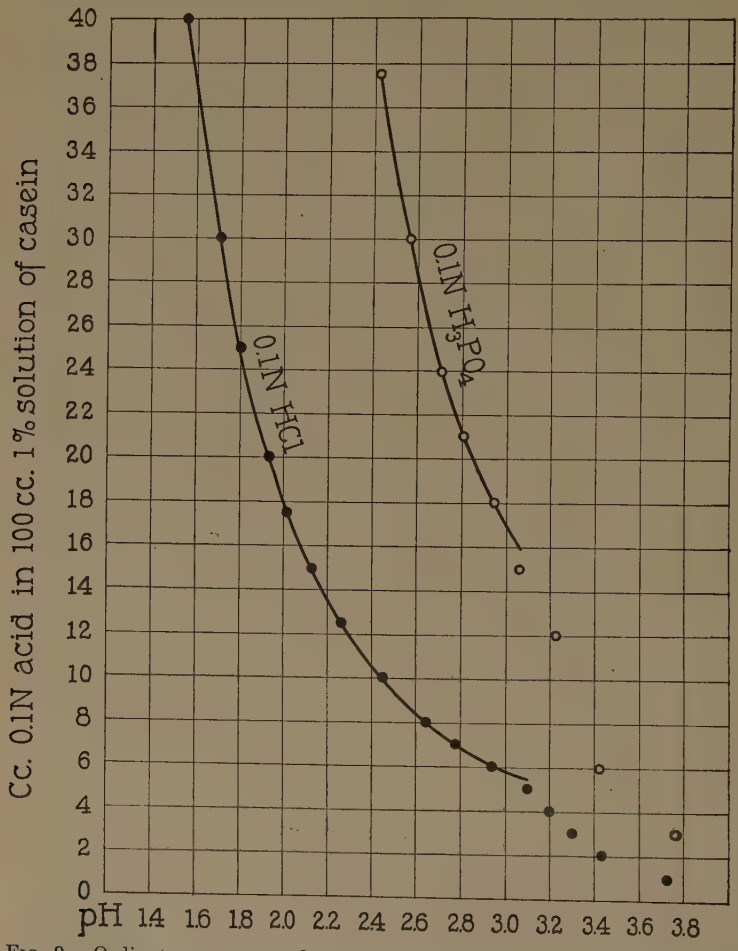

FIG. 9.—Ordinates represent the c.c. of 0.1 N HCl or H_3PO_4 in 100 c.c. of 1 per cent casein solution. The abscissæ are the pH of the solution. Approximately three times as many c.c. of 0.1 N H_3PO_4 as of 0.1 N HCl are required to bring 1 gm. of casein to the same pH.

tion to the same pH were plotted over the final pH of the casein solution as abscissæ. The casein chloride or casein phosphate is not completely soluble in a 1 per cent solution until the pH is

about 3.0 or a trifle below. When too much acid is added, *i.e.*, when the pH is 1.6 or possibly a little above, casein precipitates out again from a 1 per cent solution.

Figure 9 gives the titration curves for HCl and H_3PO_4, drawn out within those limits of pH within which the casein salts are soluble in a 1 per cent solution. The curves show that about three times as many cubic centimeters of 0.1 N H_3PO_4 as of 0.1 N HCl are required to bring 1 gm. of originally isoelectric casein in a 1 per cent solution to the same pH; or in other words, H_3PO_4 combines with casein in molecular proportions, as should be expected if casein phosphate is a true chemical compound.

It was not possible to plot the corresponding curves for casein sulphate and casein oxalate since these salts are too sparingly soluble. This is true also for casein salts with other acids, *e.g.*, trichloracetic acid.

From all these experiments we draw the conclusion that acids combine with crystalline egg albumin, gelatin, and casein (and probably proteins in general) by the same forces of primary valency by which the same acids combine also with crystalloidal substances, *e.g.*, NH_3 or NaOH.

3. In the preceding experiments we started with isoelectric protein and determined the number of cubic centimeters of 0.1 N acid required to bring the protein solution to a definite pH. It seemed of interest to confirm our results by the reverse titration; namely, by starting with a protein-acid salt of a definite pH and determining how many cubic centimeters of 0.1 N NaOH are required to bring a solution of a protein-acid salt to a definite pH, *e.g.*, 7.0. This method requires, however, certain corrections which will become clear from the following considerations. The experiments were made with gelatin solutions containing about 0.8 gm. of originally isoelectric gelatin in 100 c.c. solution. When we add different quantities of 0.1 N acid, *e.g.*, HBr, to 0.8 gm. of isoelectric gelatin, melt, and make a 0.8 per cent solution by adding enough water to bring the volume to 100 c.c., there is in solution a mixture of two substances, namely, free hydrobromic acid and gelatin bromide. The total amount of Br contained in 10 c.c. solution can be determined by titrating for Br; part of this Br is in combination with protein and part is in combination in the free HBr. The latter part can be

ascertained from the pH of the gelatin bromide solution by preparing a solution of HBr of the same pH in water, without gelatin, and determining the amount of Br in this solution free from gelatin. By deducting this value from the total Br it can be found how much HBr is combined with the gelatin. Table IV gives the results of such an experiment.[1] Row 1 gives the number of cubic centimeters of 0.01 N free hydrobromic acid originally contained in 100 c.c. of the 0.8 per cent solution of originally isoelectric gelatin. Row 2 gives the pH of each gelatin-bromide solution after equilibrium is established; Row 3 the total amount of 0.01 N Br found in 10 c.c. of the solution; and Row 4 gives the amount of Br actually in combination with gelatin after deducting the amount of Br in the free HBr (not in combination with gelatin) from the total amount of Br found.

There is a second method of ascertaining the amount of HBr in combination with a given mass of gelatin, namely by titrating for acid with NaOH.[1] In this case the number of cubic centimeters 0.01 N NaOH required to bring 10 c.c. of the gelatin-bromide solution to a pH of 7.0 must be determined. This gives the total acid, from which the value for free acid not in combination with gelatin is to be deducted. This value is obtained by titrating a solution of HBr (free from gelatin) of the same pH as the gelatin-bromide solution with NaOH. A second correction, however, must be made; namely, the quantity of NaOH required to bring 10 c.c. of an 0.8 per cent solution of isoelectric gelatin to a pH of 7.0 must be ascertained. This value was found to be about 1.8 c.c. 0.01 NaOH for 10 c.c. of a 0.8 per cent solution of isoelectric gelatin. This value must also be deducted, and if these two deductions are made, approximately the same figures are reached as by direct titration for Br. This is shown by Table IV. Row 5 gives the number of cubic centimeters 0.01 N NaOH required to bring 10 c.c. of gelatin solution to pH of 7.0. Row 6 gives the corrected NaOH values, *i.e.*, after the two deductions just mentioned are made from the values in Row 5. A comparison of the values of Row 6 with those of Row 4 shows that they are identical within the limits of the accuracy of our methods.

This method of titrating with NaOH allows us, therefore, to

[1] Loeb, J., *J. Gen. Physiol.*, vol. 1, p. 559, 1918–19.

TABLE IV.—0.8 PER CENT SOLUTION OF ISOELECTRIC GELATIN MADE UP IN 100 C.C. H_2O CONTAINING VARYING QUANTITIES OF 0.01 N HBr

1. Cubic centimeters 0.01 N HBr added	50.0	40.0	35.0	30.0	25.0	22.5	20.0	17.5	15.0	12.5	10.0	7.5	5.0	2.5	1.0	0.5
2. pH of gelatin solution	2.8	3.0	3.1	3.15	3.25	3.3	3.35	3.4	3.5	3.6	3.75	3.9	4.1	4.3	4.5	4.7
3. Cubic centimeters 0.01 N Br found in 10 c.c. of gelatin solution	8.55	7.3	6.8	6.4	5.7	5.4	4.85	4.45	4.0	3.3	3.0	2.4	1.5	0.65	0.2	0.1
4. Corrected values of Br	7.0	6.1	5.8	5.6	5.1	4.9	4.5	4.05	3.66	3.05	2.8	2.28	1.42	0.55	0.15	0.0
5. Cubic centimeters 0.01 N NaOH required to bring 10 c.c. of gelatin solution to pH 7.0	10.0	9.2	8.4	8.0	7.3	7.0	6.55	6.3	5.8	5.15	4.7	4.2	3.5	2.7	2.15	1.9
6. Corrected NaOH value	6.7	6.25	5.6	5.4	4.9	4.7	4.35	4.1	3.66	3.1	2.7	2.28	1.62	0.8	0.3	0.0

(In this experiment the collodion bags containing the gelatin solutions were put into aqueous solutions of HBr of the same concentration as that added to the gelatin; as a consequence, HBr diffused from the outside into the gelatin solution so that at equilibrium the latter contained more Br than originally added.)

find out the amount of any acid in combination with a given mass of gelatin of a certain pH.

With the new method we can also confirm the statement that weaker dibasic or tribasic acids, like oxalic or phosphoric, combine with gelatin in molecular proportions. Table V gives the equivalents of HNO_3, oxalic, and phosphoric acids in combination with gelatin at different pH in 10 c.c. of 0.8 per cent solution of originally isoelectric gelatin.

The values found for HNO_3 in Table V are slightly less than those found for HBr in Table IV and HCl, due to the fact that the concentration of gelatin was slightly less in the experiments recorded in Table V than in Table IV.[1] A comparison of the figures for NaOH values for HNO_3, and for the PO_4 values (Table V, Rows 1 and 3), found by direct titration for PO_4 with the uranyl-acetate method, shows for the two values practically the ratio of 1:3 at the same pH; *i.e.*, three times as much H_3PO_4 as HNO_3 is in combination with the same mass of gelatin. The figures for HNO_3 and oxalic acid (Rows 1 and 2, Table IV) give the ratio of approximately 1:2 for pH 3.5 or below. Hence, oxalic and phosphoric acids combine in molecular proportions with gelatin. In the same way it was shown that H_2SO_4 combines in equivalent proportions with gelatin.

These measurements confirm the conclusions at which we arrived by the other method.

TABLE V.—CUBIC CENTIMETERS OF 0.01 N ACID IN COMBINATION WITH GELATIN IN 10 C.C. OF AN 0.8 PER CENT GELATIN SOLUTION AT DIFFERENT pH

pH	3.1	3.2	3.3	3.4	3.5	3.7	3.9	4.1	4.2	4.3
1. HNO_3	4.35	4.1	3.6	3.2	2.85	2.45	1.9	1.45	....	0.75
2. Oxalic acid	9.6	8.75	7.6	6.7	6.00	4.3	3.0	....	1.65	....
3. H_3PO_4	....	12.4	10.4	9.8	9.00	7.4	5.8	4.5	2.6	2.1

[1] In these earlier experiments 1 gm. of powdered gelatin was brought to the isoelectric point. This entailed some loss, especially during the washing, which varied slightly in different experiments. In later experiments this source of error was avoided by using a stock solution of about 8 per cent isoelectric gelatin and ascertaining the concentration of isoelectric gelatin by dry-weight determinations.

It was found in these experiments that all strong monobasic acids, like HBr or HNO_3, gave the same titration curve as HCl. This, however, was, of course, no longer the case for weak acids. The weaker the acid the more is required to bring the protein solution to the same pH. This is illustrated in Fig. 10, which gives the titration curves for 0.1 N acetic, mono-, di-, and tri-

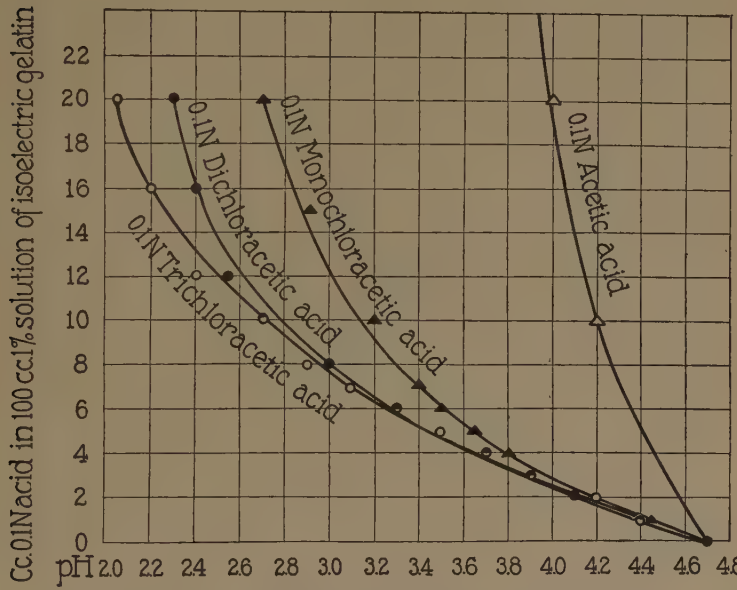

FIG. 10.—The ordinates represent the number of c.c. of 0.1 N acetic, mono-, di-, and trichloracetic acids required to bring about 0.8 gm. of isoelectric gelatin to the pH indicated by the abscissæ. Enough H_2O was added to bring the gelatin-acid solution to a volume of 100 c.c.

chloracetic acids with the same mass of isoelectric gelatin (about 0.8 gm.) in 100 c.c. solution. It is obvious that the weaker the acid the more is required to bring the same mass of isoelectric gelatin to the same pH.

On account of the enormous quantities required in the case of weak acids, it is not well possible to plot the quantity of acid in combination with a given mass of protein in the same way as done in the case of HCl; but it will be shown in the next chapter by an indirect method that the amount of anion combined with a given

mass of protein in the same volume of solution is the same for a given pH no matter whether the acid is strong or weak.

4. If the numbers of cubic centimeters of 0.1 N KOH, NaOH, $Ca(OH)_2$, and $Ba(OH)_2$ are measured which must be contained

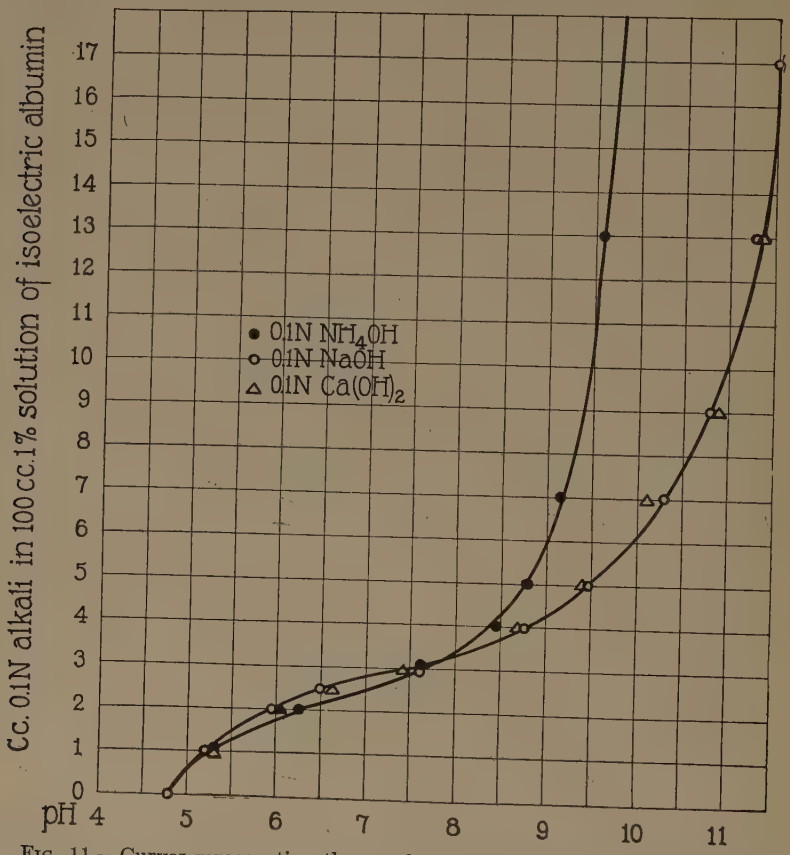

FIG. 11.—Curves representing the number of c.c. of 0.1 N NH_4OH, NaOH, and $Ca(OH)_2$ required to bring 1 gm. of isoelectric, crystalline egg albumin in 100 c.c. solution to different pH. The curves for NaOH and $Ca(OH)_2$ are identical.

in 100 c.c. of a 1 per cent solution of originally isoelectric crystalline egg albumin to bring the solution to the same pH, it is found that these numbers are identical and that the values for the four bases lie in one curve. This means that Ca $(OH)_2$ and $Ba(OH)_2$ combine

in equivalent proportions with crystalline egg albumin; *i.e.*, they combine with crystalline egg albumin in the same stoichiome-

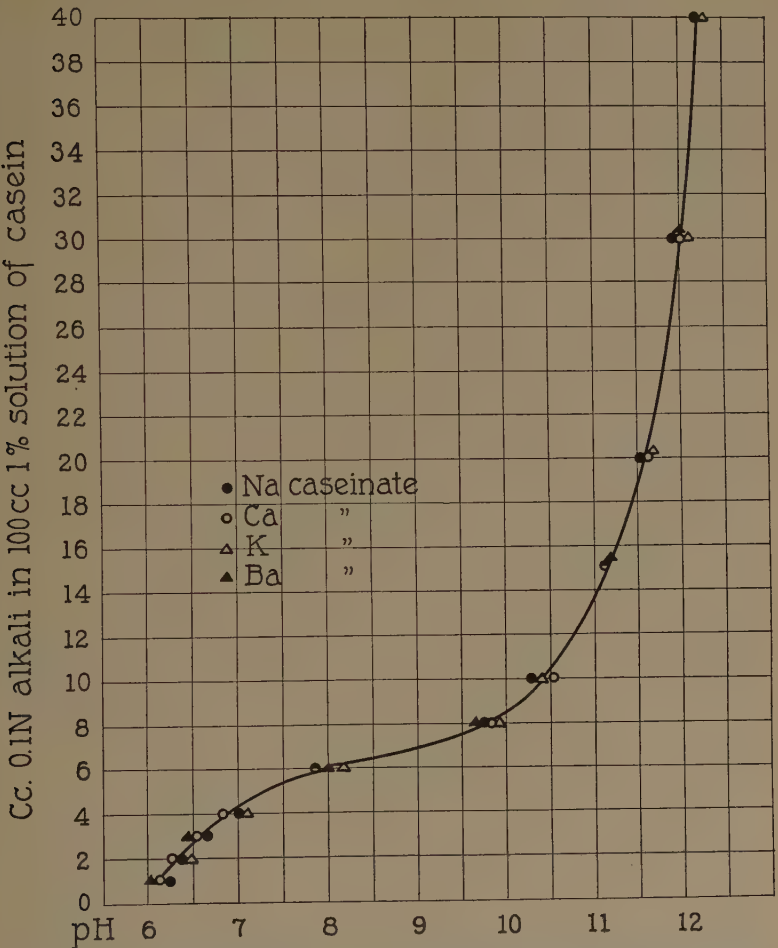

FIG. 12.—Ordinates are the c.c. of 0.1 N NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$ in 100 c.c. of 1 per cent solution of casein. Abscissæ are the pH of the solution. The curves for the four alkalies are identical, proving that Ba and Ca combine with casein in equivalent proportion.

trical way in which they combine with crystalloidal acids. This is equally true for the combination of these bases with isoelectric albumin (Fig. 11), with casein (Fig. 12), and with

gelatin (Fig. 13). In this latter case the solution contained only about 0.8 gm. of originally isoelectric gelatin in 100 cc. solution.[1]

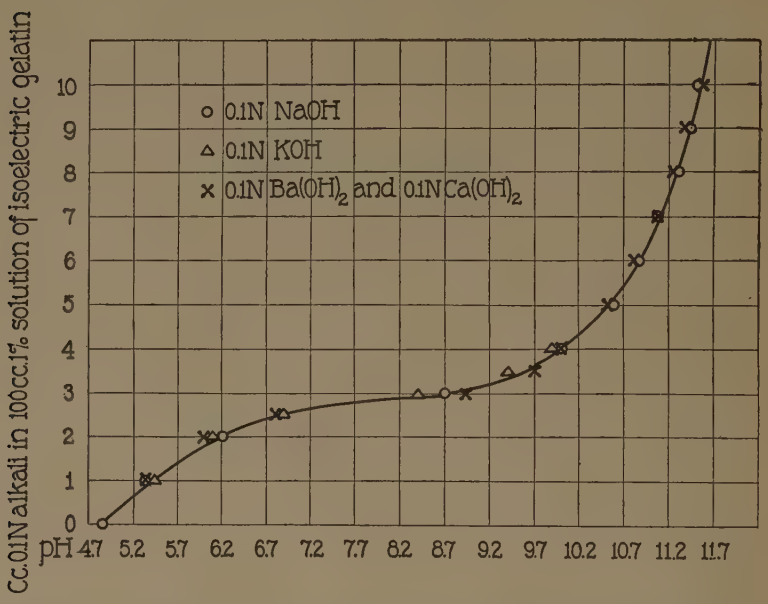

FIG. 13.—Curves for the number of c.c. of 0.1 N NaOH, KOH, $Ba(OH)_2$, and $Ca(OH)_2$ required to bring the same mass of about 0.8 gm. of isoelectric gelatin in 100 c.c. solution to different pH. All four curves are identical.

The question may be finally raised, How many molecules of acid or alkali can combine with one molecule of protein? The smoothness of the titration curves of isoelectric proteins with acids indicates that either only one or many molecules of a monobasic acid, *e.g.*, HCl, combine with one molecule of protein, since otherwise, the curves could not be smooth. It is not probable that only one molecule of acid combines with one molecule of protein.

Procter and Wilson, have reached the conclusion that the equivalent weight of gelatin is 768 (see Chap. XI), and Wintgen and Krüger[2] give the value as 839. According to the recent

[1] LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920–21.

[2] WINTGEN, R., and KRÜGER, K., *Kolloid-Z.*, vol. 28, p. 81, 1921.

analyses by Dakin,[1] gelatin contains 1.4 per cent phenylalanine, which would give as the minimal molecular weight of gelatin 11,800. Procter and Wilson's value leads to the result that about 15, or a multiple of 15, molecules of a monobasic acid combine with one molecule of gelatin.

It can be stated, as the result of all these titration experiments, that the ratios in which acids and bases combine with proteins are identical with the ratios in which acids and bases combine with crystalloids. Or, in other words, the forces by which gelatin, egg albumin, and casein (and probably proteins in general) combine with acids or alkalies are the purely chemical forces of primary valency.

The question may be raised, How can the fact that proteins combine stoichiometrically be reconciled with the statement that their solutions frequently contain aggregates of molecules? This latter fact led to the assumption of adsorption at the surface of each micella, but without cogent reason. The protein micellæ which may exist in a solution of gelatin in water are not comparable with metallic spheres or oil globules in water, where the two phases are separated by a continuous boundary. When a 1 per cent gelatin solution sets to a gel, the equal distribution of the molecules of the gel in the water remains the same. The random orientation of the gelatin molecules in the solution may change to a more definite orientation in the gel, but the average distance between the protein molecules will probably not change. The interstices between the molecules remain the same, and the protein molecules and protein ions remain as accessible to alkali or acid in the gel as are molecules or ions in true solution. The micellæ of gelatin in solution are submicroscopic particles of jelly and there is no reason why the reactions between gelatin and electrolytes should not be stoichiometrical even if the protein were entirely in the gel state.

The titration experiments given in this chapter show also why it is necessary to compare the relative efficiency of two kinds of ions of the same sign not only for the same concentration of the originally isoelectric protein but also for the same pH. As the combination curves Figs. 6 and 8 show, at each pH only part of the mass of the protein present exists in the form of a salt, the

[1] Dakin, H. D., *J. Biol. Chem.*, vol. 44, p. 499, 1920.

rest exists as non-ionogenic protein. Only if enough acid (or alkali) is added, is all the protein transformed into a salt. The combination curves show that at the same pH the same fraction of the protein present exists in the form of a protein salt. If it is desired to compare the relative efficiency of different ions in combination with a protein, one must be sure that the concentration of originally isoelectric protein is the same in both solutions and that the fraction of protein which has combined with the two ions is the same. This is only true when the solutions of the protein salts to be compared have not only the same concentration of originally isoelectric protein but also the same pH.

There existed no reason for comparing the effects of different ions at the same pH as long as the titration curves given in this chapter were not known. But the knowledge of these curves forces the experimenter to change his methods and to base all the comparisons of the influence of ions on the physical properties of proteins on protein solutions of equal hydrogen ion concentrations.

CHAPTER V

THE VALENCY RULE AND THE HOFMEISTER SERIES

(*A*) Osmotic Pressure

In this chapter it will be shown that the combining ratios of acids and alkalies with proteins furnish the key for the understanding of the influence of ions on the physical properties of proteins, inasmuch as only the sign of charge and the valency but not the other properties of an ion influence such physical qualities of proteins as osmotic pressure, viscosity, and, in the case of gelatin, swelling. In this discussion only the monovalent and bivalent ions will be considered.

The fact to be proved is contrary to the statements current in colloid chemistry according to which the chemical nature of the ion is of as much importance as the valency. As already stated in the first chapter, the ions have been arranged in series, the so-called Hofmeister series, according to their relative influence on swelling, viscosity, and osmotic pressure of proteins. It is perfectly true that the different ions of the same valency, *e.g.*, Li, Na, K, Rb, Cs, or Cl, Br, and I, have different chemical properties according to their position in the periodic table, and monovalent anions, such as NO_3, CH_3COO, also have definite chemical characteristics different from those of I or Br or Cl. These differences manifest themselves in many phenomena, *e.g.*, in solubility, but they are obviously of minor importance in their influence on the physical properties of proteins alluded to, for a reason which will become clear in the second part of the book. For the present it will only be shown that the Hofmeister ion series are largely the result of the same methodical error which had prevented the recognition of the fact that acids and alkalies combine with proteins stoichiometrically, namely, the failure to measure the hydrogen ion concentration of the protein solutions. If we wish to compare the relative efficiency of two

ions, *e.g.*, Cl and CH_3COO, on the osmotic pressure or viscosity of protein solutions, it is absolutely necessary to do so at the same pH and the same concentration of originally isoelectric protein. If this is done, it will be found that the Hofmeister series have practically no real significance and that essentially only the valency, not the specific nature of the ion in combination with the protein, influences its physical properties.

In the preceding chapter it was seen that at the same pH three times as many cubic centimeters of 0.1 N H_3PO_4 as of HNO_3 are in combination with 1 gm. of originally isoelectric gelatin in 100 c.c. of solution. From this it follows that the anion of gelatin phosphate is the monovalent ion H_2PO_4 and not the trivalent anion PO_4. It follows likewise from the combining ratios discussed in Chap. IV that the anion of oxalic acid in combination with protein below pH = 3.0 is the monovalent anion HC_2O_4, while at pH above 3.0 the oxalic acid dissociates to an increasing degree as a dibasic acid, forming a divalent anion C_2O_4 with protein. The same must be true, *mutatis mutandis*, for all weak dibasic or tribasic acids, *e.g.*, citric, tartaric, or succinic acids, namely, that at pH below 4.7 they form protein salts with chiefly monovalent anions. It follows also from the combining ratios, that the salt of a protein with a strong dibasic acid, as H_2SO_4, must have a divalent anion, *e.g.*, SO_4. On the basis of our valency rule, we should, therefore, expect that the osmotic pressure of 1 per cent solutions of originally isoelectric gelatin with different acids of the same pH should be identical for all gelatin salts with monovalent anion; in other words, 1 per cent solutions of gelatin chloride, bromide, nitrate, tartrate, succinate, citrate, or phosphate should all have about the same osmotic pressure and the same viscosity at the same pH; and the same should be true for swelling; while gelatin sulphate, which has a bivalent anion, should have a much lower osmotic pressure, viscosity, or swelling. We will show first that this is true for the osmotic pressure of protein solutions.

The simple method of R. S. Lillie[1] was employed for the measurement of the osmotic pressure of gelatin solutions. Collodion bags of a volume of about 50 c.c. were cast in Erlenmeyer flasks assuming the shape of the latter. These were prepared in a uniform way, as follows: Collodion (Merck, 275 grains of ether

[1] Lillie, R. S., *Am. J. Physiol.*, vol. 20, p. 127, 1907–08.

per ounce; 27 per cent alcohol, U. S. P. IX) was used. Erlenmeyer flasks of a volume of about 50 c.c. were rinsed with 95 per cent alcohol and then filled to the neck with the collodion solution. After the flask was filled with collodion, the latter was allowed to pour out slowly from the flask which was rotated slowly by hand during this process. The process of rotating the flask and pouring out the collodion was timed to occupy exactly 2 minutes. Then the Erlenmeyer flask, which was now empty except for a film of collodion adhering to the inside of the glass wall, was allowed to dry for exactly 2 minutes at room temperature. It was then put under the faucet and tap water was allowed to run in in a gentle stream for 5 minutes. The collodion film formed inside the flask could be pulled out being an exact cast of the flask. These collodion bags were closed, with the aid of rubber bands, by a conical rubber stopper which was perforated to allow a glass tube to be pushed through. The collodion bag was filled with the solution of protein with the aid of a small funnel, all air bubbles were removed and the glass tube was pushed into the bag to serve as a manometer. The bag was then put into a beaker usually containing 350 c.c. of water, having the same pH as the protein solution. The surface of the stopper was so adjusted that it lay in the surface of the water in the beaker and the glass tube (or manometer) was pushed a little deeper into the bag so that at the beginning the level of the protein solution was about from 20 to 30 mm. above that of the water in the outside beaker.

The water diffused from the outside beaker into the protein solution and the column of liquid in the manometer rose to a maximum which was usually reached in about 6 hours or possibly less. It must be taken into consideration that two changes in pH will occur in these experiments which affect the osmotic equilibrium. The one change is due to the Donnan equilibrium which was referred to in the introduction. The other change is due to the influence of the CO_2 of the air in the outside solution and this influence is especially disturbing when alkaline solutions are used. It must also be borne in mind that in these experiments the protein solution is also usually diluted through the entrance of water into the collodion bag. In later chapters measures will be mentioned by which these sources of error can be avoided or diminished.

One per cent solutions of originally isoelectric protein were made, each solution containing a certain amount of an acid or of alkali to give it a definite pH. In each case the collodion bag

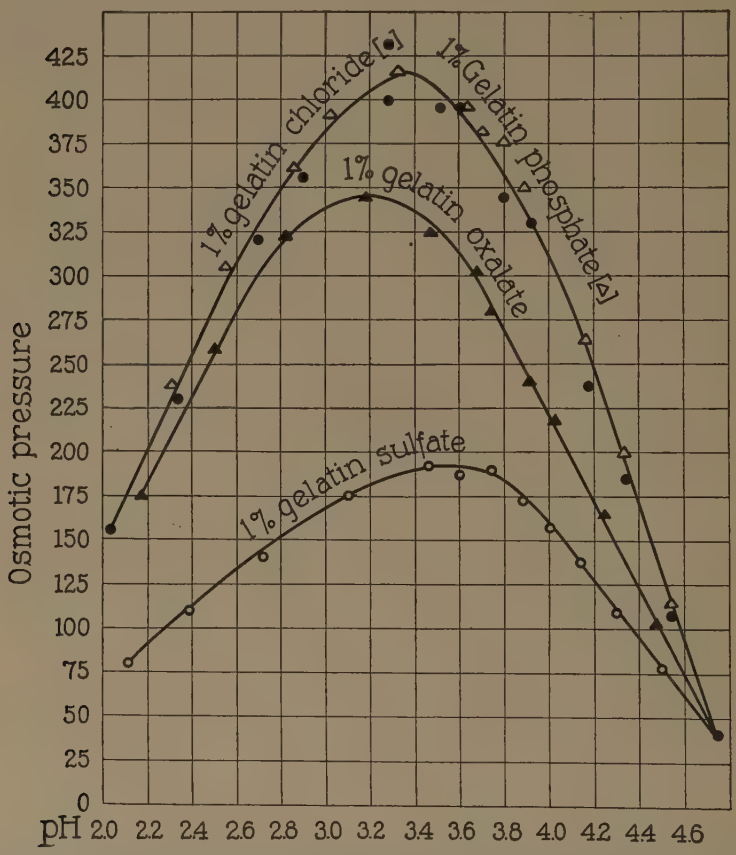

FIG. 14.—Influence of pH and valency of anion on osmotic pressure of solutions of different gelatin-acid salts. The osmotic pressure is a minimum at the isoelectric point, pH 4.7, rises with the addition of acid until pH is 3.4, and then drops upon the addition of more acid. The curves for gelatin chloride and gelatin phosphate are identical.

containing the gelatin solution was dipped, as described, into a beaker containing 350 c.c. of water of originally the same pH as that of the protein solution used. On account of the Donnan equilibrium this equality of pH in the inside and outside solu-

tions was not retained, the pH rising higher inside than outside in the case of solutions of gelatin-acid salts. The observations lasted usually for 1 day but the level of liquid in each manometer was recorded at first every 20 or 30 minutes and the values recorded the next day were used to plot the curves in Fig. 14. The osmotic equilibrium was usually established in about 6 hours. The experiments were carried on in a thermostat at a temperature of 24°C.

Figure 14 gives the curves of the osmotic pressure for solutions of originally 1 per cent isoelectric gelatin with four different acids, HCl, H_2SO_4, oxalic, and phosphoric acids.[1] The abscissæ are the pH of the gelatin solutions after osmotic equilibrium was established, *i.e.*, at the end of the experiment. The pH was always determined potentiometrically. The reader will notice that the four curves have a number of characteristic features in common. The osmotic pressure is, in all cases, a minimum at the isoelectric point, namely, at pH = 4.7; it rises with increasing hydrogen ion concentration (or diminishing pH), and the curves all reach a maximum at about pH = 3.4. When the hydrogen ion concentration rises still further (or with a further drop in pH) the curves for the osmotic pressure of the solutions of the four gelatin salts diminish almost as steeply as they rose on the other side of the maximum. It may be noticed in passing, that Pauli[2] and Manabe and Matula[3] speak of a maximum in the viscosity curves of albumin at a pH of about 2.1. It will be observed that the maximum for osmotic pressure lies at a much higher pH, namely at about pH 3.4, and that at pH 2.1 the curves are at a low level again, not much above that of the isoelectric point. This form of the curves of osmotic pressure when plotted as a function of pH of the protein solutions is very characteristic and invariable.

The main point, however, which interests us in this connection is the proof of the valency rule. The titration curves show that in the case of gelatin phosphate as well as of gelatin chloride the anion is monovalent, H_2PO_4 and Cl. The valency rule demands

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 691, 1920–21.

[2] Pauli, W., "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

[3] Manabe, K., and Matula, J., *Biochem. Z.*, vol. 52, p. 369, 1913.

that the osmotic pressures of the two salts should be identical and a glance at Fig. 14 shows that this is the case. The anion of gelatin oxalate should also be essentially monovalent for pH below 3.0 and we see that the descending branch of the oxalate curve, from pH 3.0 and below, practically coincides with the descending branch of the curve for gelatin chloride and phosphate. For pH above 3.0 the curve for the osmotic pressures of gelatin oxalate is slightly lower than the curve for gelatin phosphate and gelatin chloride, as the theory of electrolytic dissociation demands, since for pH above 3.0 oxalic acid dissociates electrolytically more and more like a dibasic acid the higher the pH. Hence, at about pH 3.4 the majority of the anions of gelatin oxalate is monovalent, but a certain small percentage is divalent. For this reason the curve for gelatin oxalate is at pH 3.4 or for higher pH not quite as high as that for gelatin chloride or phosphate. This is in strict agreement with the titration curves in Fig. 7.

The titration curves in Fig. 7 show also that H_2SO_4 forms a divalent anion in combining with gelatin and we notice that the maximum of the osmotic pressure curve at pH 3.4 is less than one-half that of the osmotic pressure curve for gelatin chloride or gelatin phosphate at the same pH.

These results are then in full agreement with the titration experiments if we assume that only (or chiefly) the sign and the valency of the ion with which the protein is in solution determine the osmotic pressure of the protein salt formed, while the nature of the ion has either no effect or if it has any effect the latter must be so small that it escapes detection.

If the Hofmeister series were correct, we should have expected that the curve for the osmotic pressure of gelatin phosphate should have been of the order of that of gelatin sulphate or even lower instead of being equal to that of gelatin chloride; and the same should have been true for the curve for gelatin oxalate.

I have repeated these experiments so often that there can be no doubt about the correctness of the result.

The experiments with 1 per cent solutions of originally isoelectric crystalline egg albumin confirm the valency rule also for this salt.[1] The abscissæ are again the pH determined at the

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920–21.

beginning of the experiment, the ordinates the osmotic pressure after equilibrium was reached. The acids used were HCl, H_2SO_4, oxalic acid, and H_3PO_4 (Fig. 15). The reader notices again that the osmotic pressures are a minimum at the isoelectric point, that they reach a maximum at pH a little above pH 3.2, and that they then drop again.

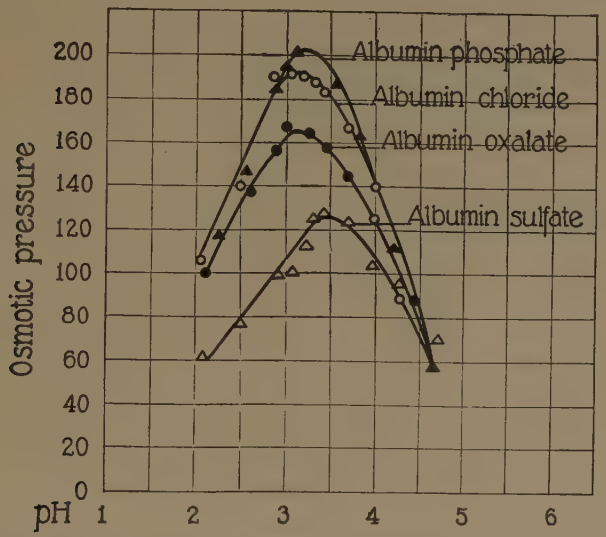

FIG. 15.—Osmotic pressure of different albumin-acid salts. The ordinates indicate the osmotic pressure (in mm. of 1 per cent albumin solution); the abscissæ are the pH. All solutions are 1 per cent in regard to isoelectric albumin. The curves for albumin chloride and albumin phosphate are identical.

The four curves confirm the valency rule. The curves for albumin chloride and albumin phosphate are practically identical, that for albumin sulphate is almost but not quite half as high as that of phosphate, and the curve for oxalate is at the maximum a little lower than that for chloride.

The valency rule holds also for casein-acid salts.[1] Since casein oxalate and sulphate are too sparingly soluble we can only compare the osmotic pressures of casein phosphate and casein chloride. The curves for the osmotic pressures of these two

[1] LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 547, 1920–21.

salts are alike if plotted over the pH, as Fig. 16 shows. The maximal osmotic pressure lies at pH of about 3.0.

There is then no doubt that the curves for the osmotic pressures of the three proteins, gelatin, crystalline egg albumin, and casein obey the valency rule, and show no appreciable influence of the nature of the ion except that of the sign of charge and valency.

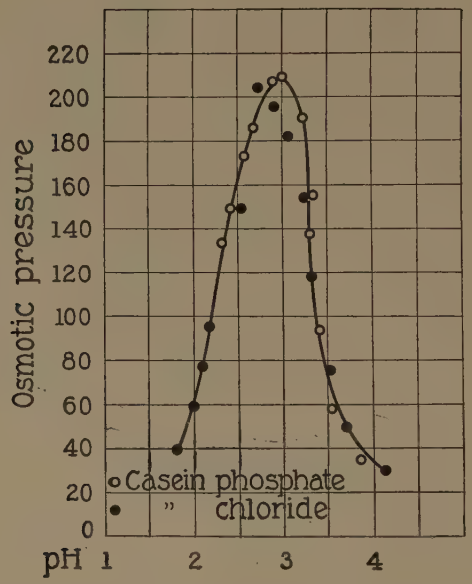

FIG. 16.—Osmotic pressure of 1 per cent solutions of casein chloride and casein phosphate as function of pH. The two curves are almost identical.

In the older experiments in which the hydrogen ion concentrations were not measured, the action of weak acids led the investigators into error. In the Hofmeister series it is generally contended that acetic acid acts like sulphuric acid and not like hydrochloric or nitric acids. This is due to the fact that the investigators compared the effects of different acids at equal molecular concentrations instead of comparing the effects of different acids at the same pH. If this is done it is found that acetic acid acts like HCl and not like H_2SO_4. Figure 17 gives the curves for the osmotic pressure of about 0.8 per cent solutions

of originally isoelectric gelatin with six different acids, HCl, H_2SO_4, acetic, monochloracetic, dichloracetic, and trichloracetic acids plotted over pH as abscissæ.[1] Since the concentration of

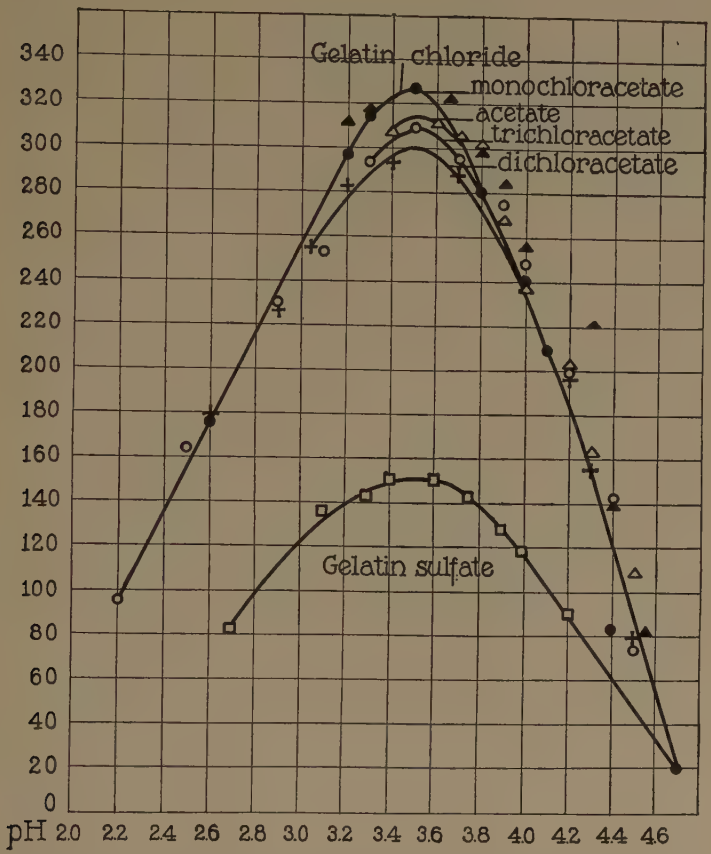

FIG. 17.—Osmotic pressure (in mm. H_2O) of about 0.8 per cent solutions of gelatin chloride, gelatin acetate, monochloracetate, dichloracetate, and trichloracetate. The curves are practically identical.

the originally isoelectric gelatin in these solutions was lower than in the experiments represented in Fig. 14 (about 0.8 per cent instead of 1 per cent), the osmotic pressures are also all lower, but the results are relatively the same. Thus the maximal osmotic

[1] LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920–21.

pressure of gelatin sulphate is in Fig. 17 also a little less than one-half of the maximal osmotic pressure of gelatin chloride and the maximum lies again at pH of about 3.4. The four acetic acids have their maximum also at the same pH and this maximum is equal to that of HCl.[1] The slight variations in the height of the curves for the five monobasic acids are merely accidental and probably chiefly due to slight differences in the concentration of the isoelectric gelatin. In these experiments each gram of powdered gelatin was brought independently to the isoelectric point and in this procedure about 20 per cent of gelatin were lost, but the loss varied slightly in the different experiments. In the experiments represented in Fig. 14 a large quantity of powdered gelatin was brought to the isoelectric point and doses of 1 gm. of isoelectric gelatin were used. In this latter case the quantity of originally isoelectric gelatin was always the same.

It was also found that the osmotic pressure of 0.8 per cent solutions of gelatin tartrate and gelatin citrate is approximately the same as that of gelatin chloride of the same pH.

The writer has also shown that the curves for the osmotic pressure of 1 per cent solutions of originally isoelectric crystalline egg albumin are identical for albumin chloride, albumin acetate, and albumin dichloracetate, when plotted over the pH as abscissæ.[1]

These experiments on gelatin and albumin leave no doubt that the acetates behave like chlorides and not like the sulphates. Pauli claimed that trichloracetic acid acted like sulphuric acid but this is certainly not the case as far as the osmotic pressure of gelatin solutions is concerned.

The idea that the valency of the ion in combination with a protein is the chief if not the only factor which influences its osmotic pressure is corroborated by measurements of the osmotic pressure of metal gelatinates. We had shown in Chap. IV that $Ca(OH)_2$ and $Ba(OH)_2$ combine with gelatin in equivalent proportions and that hence, the ion in combination with gelatin in these cases is the bivalent cation Ca or Ba. The experiments showed that Li, Na, K, and NH_4 gelatinate have about the same

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920–21.

osmotic pressure at the same pH and the same concentration of originally isoelectric gelatin; while under the same conditions Ba and Ca gelatinate have an osmotic pressure less than one-half of that of the metal gelatinates with monovalent cation.[1] The same

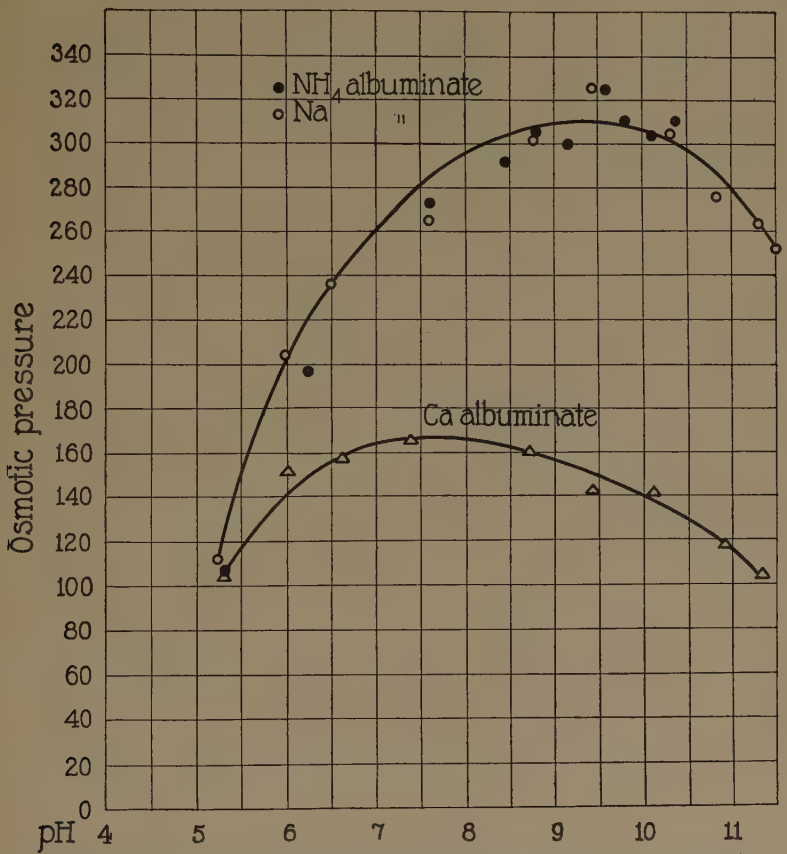

FIG. 18.—Curves of the osmotic pressure of NH_4, Na, and Ca albuminate at different pH. The curves for NH_4 and Na albuminate are practically identical.

is true in the case of the metal salts of crystalline egg albumin. Figure 18 shows that the curves for the osmotic pressure of NH_4 and Na albuminate are about the same for the same pH while that for Ca albuminate is about half as high.

[1] LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 85; 547, 1920–21.

Similar results were obtained in the case of the osmotic pressure of metal caseinates.

All experiments agree that only the sign and the valency of the ion with which a protein is in combination determine its osmotic pressure while the specific nature of the ion seems to have no influence. This fact is of the greatest significance since it was to be expected if colloidal behavior is due to the Donnan equilibrium. The writer may state that this valency rule was found before he was aware of the fact that the influence of electrolytes on the osmotic pressure of protein solutions could be derived from the Donnan equilibrium.

(*B*) Swelling

It is generally stated in colloidal literature that solid blocks of gelatin swell more in chlorides, bromides, or nitrates than in water and that they swell less in citrates, acetates, tartrates, phosphates, and sulphates than in water. The author of this statement is Hofmeister[1] who was a pioneer and who cannot be blamed for not considering the hydrogen ion concentration of his solutions about which nothing was known at the time of his experiments. In Hofmeister's experiments gelatin blocks were put into salt solutions of a high concentration, and the differences in the effects observed in different solutions were slight.

He even states that sugar solutions have a dehydrating effect, like certain salts, and this fact alone should have warned chemists that his experiments could not be used for conclusions concerning the specific effects of ions on the physical properties of colloids. As far as the writer knows the discrimination between "hydrating" and "dehydrating" ions originated from these experiments.

It is often asserted that Hofmeister's ion series for swelling have been confirmed by other authors. Thus on page 373 of Zsigmondy's book "Kolloidchemie" (2nd edition), the following statements are made in support of this impression.

"Wo. Ostwald who compared the efficiency of different acids found that swelling diminishes in the acids in the following order, HCl > HNO_3 > acetic acid > sulphuric acid > boric acid. Fischer has shown that the acid and alkali swelling of gelatin as well as that of fibrin is

[1] Hofmeister, F., *Arch. exp. Path. u. Pharm.*, vol. 28, p. 210, 1891.

diminished by the addition of salt, and that chlorides, bromides, and nitrates have a less dehydrating action than acetates, sulphates, or citrates. We have here a similar series as in the case of the precipitation of proteins by alkali salts, although the order does not agree entirely."

The writer is inclined to interpret Ostwald's and Fischer's experiments differently from Zsigmondy, since both authors ignored the hydrogen ion concentration of their solutions. Our experiments have shown that it is necessary to base conclusions concerning the relative efficiency of ions on experiments with equal hydrogen ion concentration. By ignoring this postulate Ostwald only proved that acetic and boric are weaker acids than nitric but not that the acetate and borate anions have a greater depressing effect on the swelling of gelatin than NO_3; and Fischer only proved that citrates and acetates are buffer salts which when added to a solution of a strong acid diminish its hydrogen ion concentration, but not that the acetate and citrate anions have a greater depressing effect on the swelling of gelatin than Cl or NO_3. Both authors erroneously ascribed the effects of variation of pH to an effect of the nature of the anion. The Hofmeister series of ion effects on swelling has, in reality, never been confirmed.

If we wish to study the specific effects of ions on the swelling of gelatin we must proceed from isoelectric gelatin, bringing it to different pH by different acids or alkalies and then compare the swelling at the same pH for these different acids or alkalies. If this is done it is found that when gelatin is in combination with the anion of a weak dibasic or tribasic acid, *e.g.*, tartaric, citric, phosphoric, its degree of swelling is the same as when it is in combination with Cl or NO_3; since in all these cases the anion of the gelatin salts is monovalent, and that only in the case of gelatin sulphate is the swelling considerably less, because H_2SO_4 combines with gelatin in equivalent and not in molecular proportions as does the weak dibasic or tribasic acid, *e.g.*, phosphoric.[1]

The following simple and quick volumetric method for measuring the swelling was adopted.

Dry powdered gelatin was sifted and the grains no longer going through sieve 50 but going through sieve 40 or 30 were

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 247, 1920–21.

selected for the experiment. Doses of 1 gm. each were weighed out and each was put for an hour into 100 c.c. M/128 acetic acid at 10°C. to bring the gelatin to the isoelectric point. The powdered mass was then put on a filter and washed five times with 25 c.c. of distilled water of 5°C. In the acetic acid solution and during the washing on the filter the powdered gelatin is stirred constantly. In this washing about 20 per cent of the gelatin were lost, so that the mass of gelatin in the following experiments was only about 0.8 gm. each.

Each dose of originally 1 gm. of dry powder which had meanwhile absorbed a certain quantity of liquid (which was about the same for each dose of isoelectric powder) was then put for 1 hour at about 20° into 100 c.c. of different concentrations of the acid or base whose influence on swelling was to be tested, and the mass was frequently agitated. To measure the relative amount of swelling in different acids or alkalies and at different pH the mass was poured into graduated cylinders of 100 c.c. in which the granules settled very rapidly to the bottom. The cylinders were kept in a water bath at 20° for about 10 to 15 minutes and the volume occupied by the gelatin granules, after settling, was then read. This volume included a certain amount of solution between the granules and, therefore, the real volume of the gelatin was smaller than that read. While therefore, the method cannot be used to measure the absolute amount of swelling it allowed us to determine the relative influence of different acids or bases on the swelling for the same pH.

The pH inside the gelatin granules and the surrounding solution are quite different, owing to the Donnan equilibrium. It is, therefore, not correct to assume that the pH of the granules of gelatin is that of the supernatant liquid. The pH of the granules of gelatin was determined after the gelatin had been poured on a filter and the acid in the interstices of the granules of gelatin had been allowed to drain off. Traces of this outside acid remained undoubtedly at the surface of the granules. The gelatin was then melted and its volume brought to 100 c.c. by adding enough distilled water of pH 5.6. The pH was determined potentiometrically. This pH was probably a trifle too low on account of some of the acid adhering.

Figures 19 and 20 give the results of the measurements of swell-

ing in acid. The abscissæ are the pH found in the gelatin after equilibrium was established. The ordinates represent the figures for the volume of the granules of about 0.8 gm. of gelatin in different acids. It is obvious that in all cases the volume (or swelling) is a minimum at the isoelectric point pH = 4.7, that it rises with diminishing pH until the maximum is reached at a pH of about 3.2 or a little less, and that the curve drops steeply with

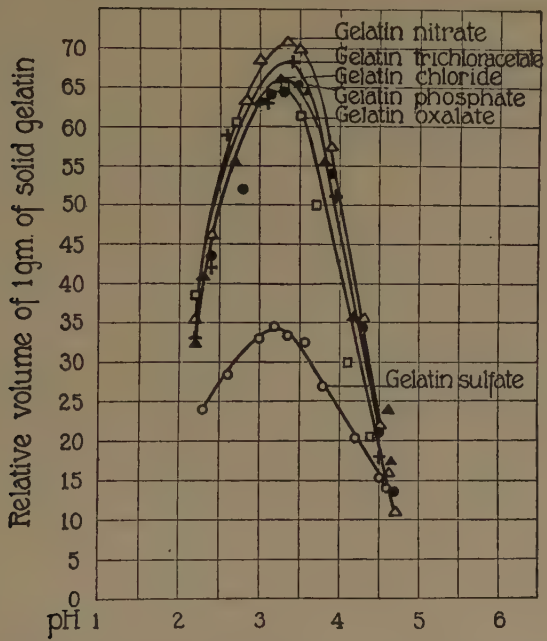

FIG. 19.—Influence of HCl, HNO_3, H_3PO_4, H_2SO_4, trichloracetic, and oxalic acids on the swelling of gelatin. Abscissæ are the pH, ordinates the volume of gelatin. The curves for all the acids are practically identical except that for H_2SO_4 which is about one-half as high as the curves for the other acids.

a further diminution of pH (*i.e.*, a further increase of hydrogen ion concentration). The main fact is, however, that the curves for the influence of HCl, HNO_3, trichloracetic, oxalic, phosphoric, citric, and tartaric acids are practically identical, proving that only the valency and not the nature of the anion of the acid used influences the swelling of gelatin; since the anion of weak dibasic or tribasic organic acids combining with the gelatin is always monovalent.

The curve for the swelling of gelatin sulphate, where the anion combining with gelatin is bivalent, is only about half as high as the curve for the salts of gelatin with the anion of weak dibasic acids (Figs. 19 and 20).

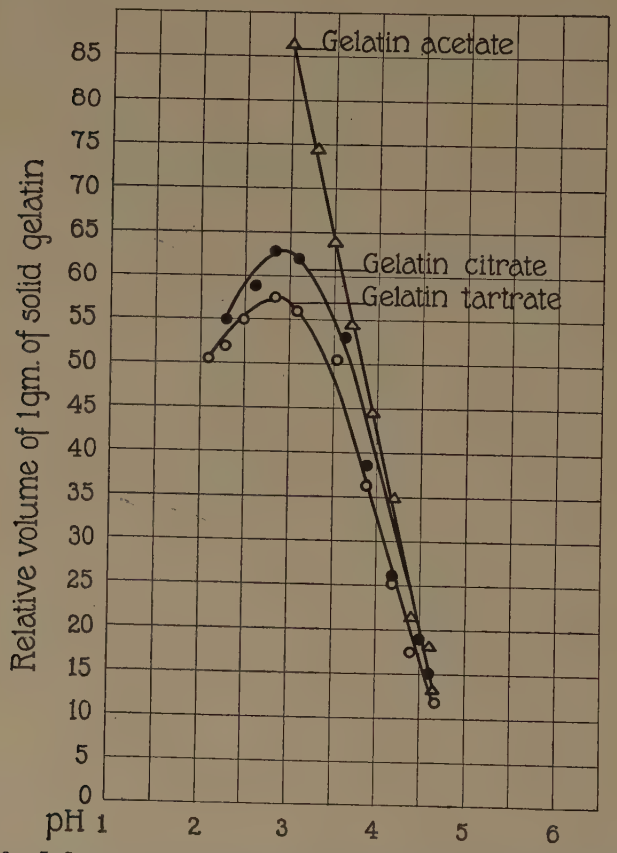

FIG. 20.—Influence of citric, tartaric, and acetic acids on swelling of gelatin. The curves for citric and tartaric acids are practically identical with those for HCl and HNO_3 in Fig. 19. That for acetic acid is a little higher owing possibly to some specific and secondary effect of this acid on the cohesion of the jelly.

Acetic acid gives an increasing amount of swelling, but it must be remembered that 1 M acetic acid had to be used to bring the pH of the gelatin to 3.0, and it is not impossible that in this case the high concentration of undissociated acid caused a secondary

physical modification of the gelatin (*e.g.*, diminution of cohesion between the particles of gelatin).

Figure 21 gives the curves for the action of alkalies on swelling. The curves for Li, Na, K, and NH_4 gelatinate of the same pH are practically the same, except that the values for NH_4OH are irregular for pH above 8.5, possibly on account of the fact that the concentration of NH_4OH required to bring gelatin to such pH

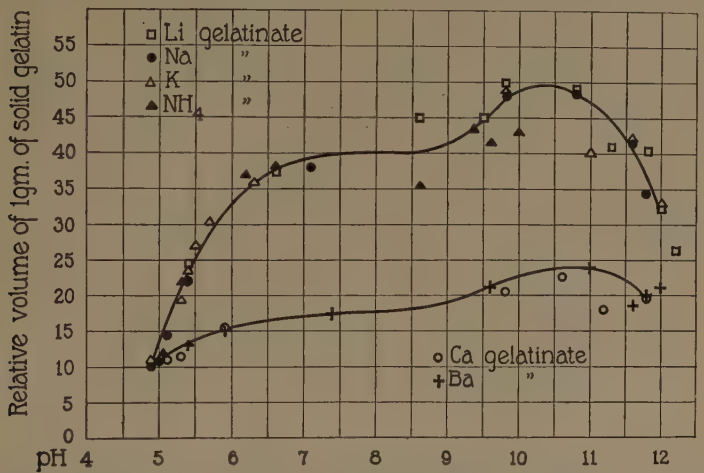

FIG. 21.—Curves for the effect of different bases on swelling. Those for LiOH, NaOH, KOH, and NH_4OH are practically identical and about twice as high as those for $Ca(OH)_2$ and $Ba(OH)_2$.

is rather high. The main fact is that the ratio of the maximal swelling of gelatin salts with bivalent cation, like Ca or Ba, is considerably less than that of gelatin salts with monovalent cation, like Na, K, or NH_4.[1] This agrees with the results of the titration experiments which show that $Ca(OH)_2$ and $Ba(OH)_2$ combine with gelatin in equivalent proportions and that, hence, the cation in combination with the gelatin is, in this case, bivalent.

It should be pointed out that the maximal swelling of gelatin in alkalies was less than that in acids. This was not observed in the osmotic pressure curves. It is probably due to differences of cohesion of the ions of the gel in the two cases.

The results show clearly that the Hofmeister series is not the correct expression of the relative effect of ions on the swelling of

[1] LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 247, 1920–21.

gelatin, and that it is not true that chlorides, bromides, and nitrates have "hydrating" and acetates, tartrates, citrates, and phosphates "dehydrating" effects. If the pH of the gelatin is taken into consideration, it is found that for the same pH the effect on swelling is the same for Cl, NO_3, trichloracetate, tartrate, succinate, oxalate, citrate, and phosphate, while the swelling is considerably less for SO_4. This is exactly what we should expect according to the valency rule on the basis of the combining ratios of different acids with gelatin, since the weak dibasic and tribasic acids combine with gelatin in molecular proportions while the strong dibasic acid H_2SO_4 combines with gelatin in equivalent proportions. In the case of the weak dibasic acids the anion in combination with gelatin is monovalent and in the case of the strong H_2SO_4 it is bivalent. Hence, it is only the valency and not the nature of the ion in combination with gelatin which affects the degree of swelling.

(*C*) Viscosity

The valency rule which permits us to predict the relative osmotic pressure of solutions of protein holds also in the case of viscosity of gelatin and casein solutions.

We will begin with experiments on the influence of gelatin on the viscosity of water.[1] A 4 per cent stock solution of isoelectric gelatin was prepared, and some of the stock solution was heated to 45° and made up to a 1.6 per cent solution in quantity sufficient for a day's experiments. This 1.6 per cent solution was kept during the day at 24°C. To 50 c.c. of this solution was added the desired acid or alkali in sufficient quantity and then the volume raised to 100 c.c. by the addition of enough distilled water. The 0.8 per cent solution was then rapidly brought to a temperature of 45°, kept there for 1 minute and was then rapidly cooled to 24°C. The solution was stirred constantly during the heating and cooling. The viscosity was measured immediately after the solution was cooled to 24°C. The measurements were all made at 24°C. by using the time of outflow through a viscometer. The time of outflow of distilled water through the Ostwald viscometer used was exactly 1 minute at

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 85, 1920–21.

24°C. Each measurement of viscosity was repeated with the same gelatin solution and the beginning and the end of a series

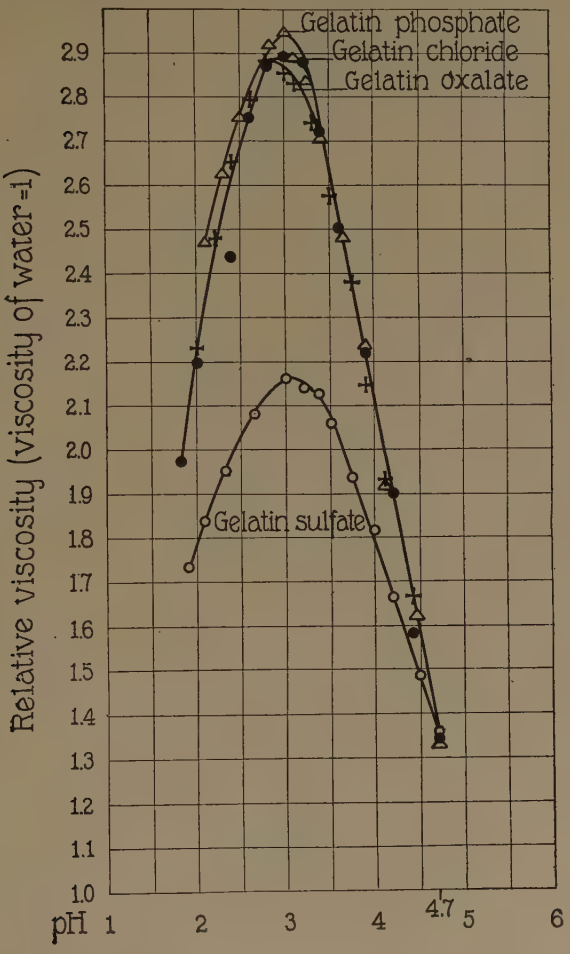

FIG. 22.—Curves representing relative viscosity of 0.8 per cent solution of originally isoelectric gelatin brought to different pH. The curves for relative viscosity of gelatin chloride, phosphate, and oxalate are practically identical. Relative viscosity is given as time of outflow of gelatin solution divided by time of outflow of water through viscometer at 24°C.

consisted in the measurement of viscosity of isoelectric gelatin. These latter measurements agreed in all experiments within 1

second varying only between 80 and 81 seconds, thus guaranteeing the reproducible character of the experiment.

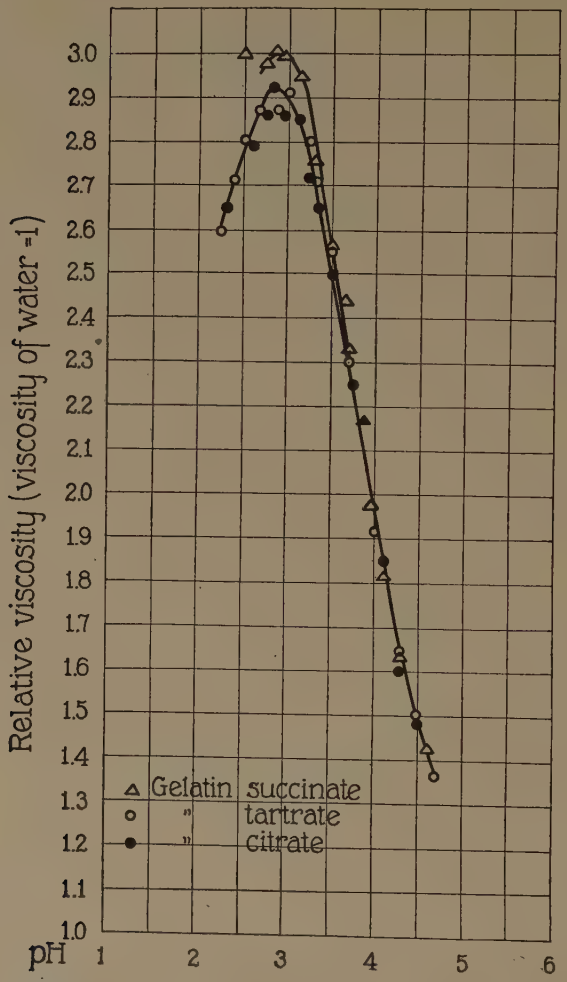

FIG. 23.—Curves representing relative viscosity of gelatin succinate, tartrate, and citrate. The curves are practically identical with those for the viscosity of gelatin chloride and phosphate.

The results can be given briefly. Figure 22 gives the curves for the relative viscosity of 0.8 per cent solutions of gelatin chloride,

sulphate, oxalate, and phosphate. The abscissæ are the pH of the gelatin solutions, the ordinates the ratio of the time of outflow of the gelatin solutions divided by the time of outflow of pure

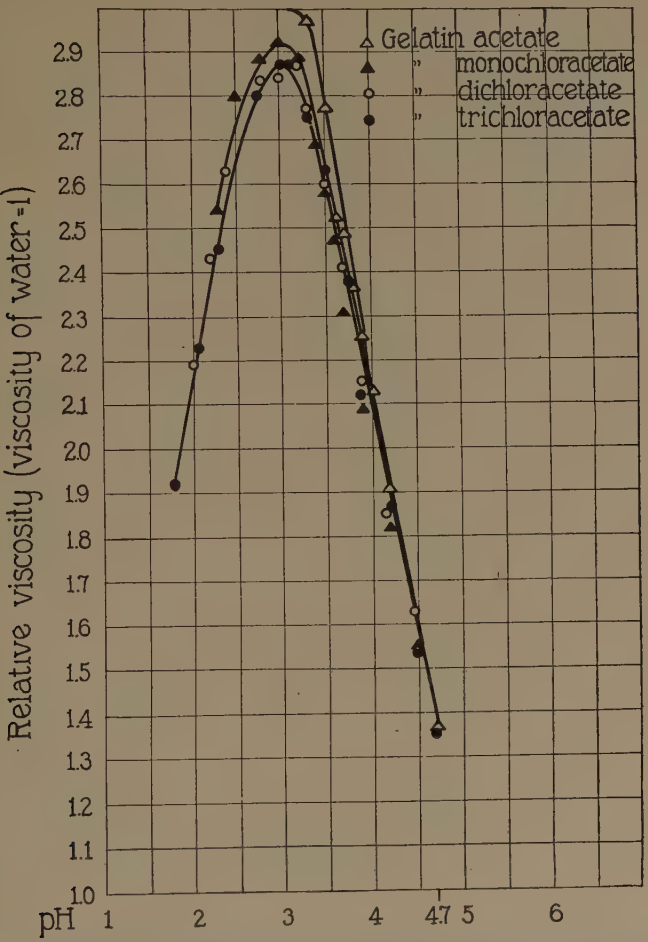

FIG. 24.—Curves representing relative viscosity of gelatin acetate, mono-, di-, and trichloracetate. Curves identical with those for gelatin chloride and phosphate.

water. For the sake of brevity this quotient will be called the relative viscosity of the gelatin solution. The curves for the four acids all rise steeply from the isoelectric point with increasing

hydrogen ion concentration until they reach a maximum at pH about 3.0 or slightly above. The curves then drop again. The curves for the three salts, gelatin chloride, oxalate, and phosphate are practically identical while the curve for gelatin sulphate is considerably lower.

Figure 23 gives the curves for the viscosity of gelatin citrate, tartrate, and succinate. The three curves are practically identical and also identical with the curves for gelatin chloride and gelatin phosphate in Fig. 22.

Figure 24 gives the curves for the viscosity of 0.8 per cent solutions of originally isoelectric gelatin to which acetic and mono-, di-, and trichloracetic acids have been added. The curves are again identical with those for gelatin chloride, phosphate, etc.

The titration curves with alkalies have shown that Ca and Ba combine with proteins in equivalent proportions and we should hence expect that the viscosity curves for Ba and Ca proteinates would be lower than those for Li, Na, K, and NH_4 proteinates. This was found to be correct.

In experiments on the viscosity of casein solutions the limited degree of solubility of the salts of casein has to be considered. In the region from 4.7 to 3.0 or even a trifle below neither casein chloride nor casein phosphate is sufficiently soluble to permit the preparation of a 1 per cent solution, and in this region the influence of casein on the viscosity of water is, therefore, negligible. The curve representing the relative viscosity of 1 per cent casein chloride and phosphate solutions (as compared with that of pure water) rises sharply at pH 3.0. With a further increase of the hydrogen ion concentration the curve falls steadily as it did in the case of the curve for gelatin. This indicates that the maximum for the influence of casein chloride on viscosity lies at pH equal to or greater than 3.0. The curve for the influence of casein phosphate on viscosity coincides with the curve for casein chloride.

The difference between the viscosity curve of Na caseinate and Ba caseinate (Fig. 25) is also similar to that of the corresponding gelatin salts.

In the influence of monovalent or bivalent ions on those physical properties of proteins which are characteristic for colloidal behavior only the valency and the sign of charge of the ion play a role,

while ions of the same sign of charge and valency have similar effects. In the second part of the book it will be shown that this is due to the fact that the colloidal behavior is the expression of the forces set up by the Donnan equilibrium and in the equation

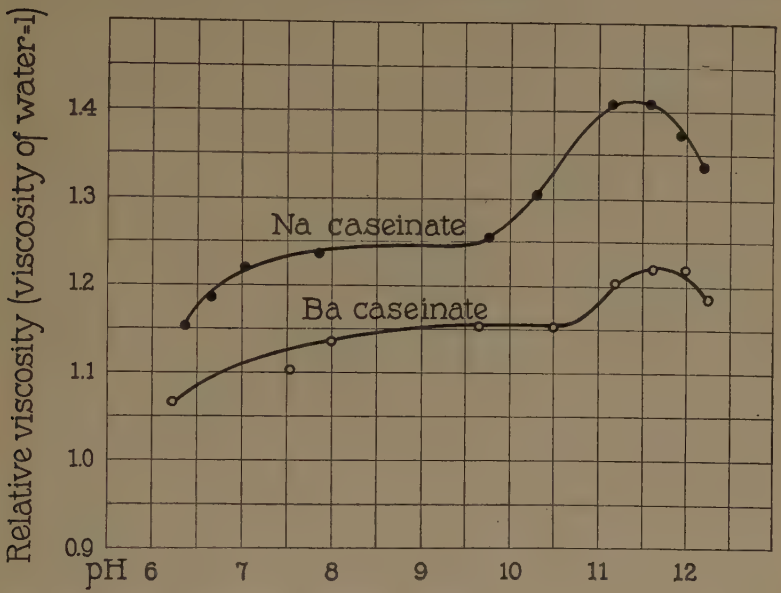

FIG. 25.—Curves representing relative viscosity of Na and Ba caseinate for different pH.

for this equilibrium only the sign of charge and valency appear. It is also obvious that it would have been impossible to arrive at this valency rule without the proof of the stoichiometrical character of the combination of proteins with acids or bases, especially the proof that weak dibasic and tribasic acids combine in the range of pH concerned in molecular proportions.

CHAPTER VI

THE ACTION OF NEUTRAL SALTS ON THE PHYSICAL PROPERTIES OF PROTEINS

1. The Difference in the Effect of Acids, Alkalies, and Salts on Proteins

The most striking proof for the alleged existence of specific ion effects on proteins (aside from those due to valency of the ion), seemed to have been furnished by experiments on the influence of neutral salts on the osmotic pressure, swelling, and the viscosity of protein solutions.

It has been noticed by a number of authors that the influence of neutral salts on the physical properties of proteins differs from that of acids and bases, and various attempts have been made to find an accurate expression for this difference. Some hold that neutral salts form "adsorption compounds" with "electrically neutral," *i.e.*, non-ionized, protein molecules, in which both ions of the salt were believed to be simultaneously adsorbed by the "neutral" protein molecule.[1] This idea is no longer tenable for salt solutions of low concentration since the experiments with powdered gelatin discussed in Chapter II have shown that only one (or practically only one) of the two ions of a neutral salt can combine at one time with a protein. At the isoelectric point, *i.e.*, at pH 4.7, gelatin can combine with neither ion of a neutral salt; at a pH > 4.7 only the metal ion of the neutral salt can combine with the gelatin, forming metal gelatinate; at a pH < 4.7 only the anion of the neutral salt is capable of combining with the protein, forming gelatin-acid salts.

R. S. Lillie has made the statement that while acids and alkalies increase, salts depress the osmotic pressure of gelatin.[2] This statement, while it was the expression of facts actually observed

[1] Pauli, W., *Fortschr. naturwiss. Forschung*, vol. 4, p. 223, 1912.

[2] Lillie, R. S., *Am. J. Physiol.*, vol. 20, p. 127, 1907–08.

by Lillie, is not entirely correct owing to the fact that the influence of the hydrogen ion concentration of the gelatin solution was not taken into consideration. It was shown in the preceding chapter that if acid is added to a gelatin-acid solution of a pH of 3.0 or below, the effect is practically the same as when we add a neutral salt, namely, a diminution of the osmotic pressure of the solution; and that when alkali, *e.g.*, KOH, is added to a solution of a metal gelatinate of pH 11.0 or above, the effect is also a similar depression of the osmotic pressure to that caused by the addition of KCl. A depression is also noticed when some acid is added to a solution of metal gelatinate or when some alkali is added to gelatin-acid salts; since in both cases the gelatin is brought nearer to the isoelectric point.

It is also incorrect to speak of an antagonism between the effects of acids and salts, since the facts mentioned show that there is also an antagonism between little and much acid; thus, if the pH of a gelatin-acid salt is 3.0, a further addition of the same acid depresses the osmotic pressure or viscosity. The question then arises, What is the correct expression of the facts in the case?

The answer seems to be as follows: Suppose the pH is below but near that of the isoelectric point of a protein and HCl be added. In this case the more acid is added the more non-ionogenic protein is transformed into salt. This salt formation raises the osmotic pressure, swelling, and viscosity of the protein. This agrees with the views of Laqueur and Sackur, and of Pauli. At the same time the anion of the acid has an opposite, namely a depressing effect. The addition of acid has, therefore, two opposite effects on the osmotic pressure, viscosity, and swelling of protein, namely, first, an augmenting effect due to increasing protein-salt formation with increasing hydrogen ion concentration, and second, a depressing effect due to the anion, in our example Cl. At first, the augmenting effect increases more rapidly than the depressing effect. When, however, the pH of the protein solution approaches the value 3.0 the augmenting influence due to the formation of new gelatin chloride grows less rapidly with a further decrease in pH than does the depressing effect of the anion, and hence, when the amount of acid added increases still further, the depressing effect of the Cl ion prevails over the

augmenting effect of the H ion. The true reason for this will appear in Chap. VIII.

When an alkali, *e.g.*, NaOH, is added to a protein, *e.g.*, gelatin, with a pH slightly above 4.7, at first more of the non-ionogenic protein is transformed into metal proteinate, *e.g.*, Na gelatinate; and this raises the osmotic pressure, viscosity, and swelling rapidly by causing an increase in the concentration of ionized protein for a reason which will be given later. The cation of the alkali, the Na ion, has a depressing effect on these properties, and this depressing effect begins to be visible when the pH exceeds a certain value. After this, with a further addition of alkali, the depressing action of the cation (*e.g.*, of Na) increases more rapidly than the augmenting action of the OH ion.

The addition of neutral salts of a concentration below N/16 to isoelectric gelatin has no effect on osmotic pressure, viscosity, or swelling of the gelatin solution. When neutral salt is added to a gelatin solution on either side of its isoelectric point only a depressing action of that ion which has the opposite sign of charge to the protein ion is observed. No augmenting action of the ion with the same sign of charge as the protein is noticeable. Thus, if $CaCl_2$ or Na_2SO_4 is added to a solution of gelatin chloride or gelatin nitrate only a depressing effect of the Cl or SO_4 ion is observed but no augmenting effect of the Ca or Na ion; while when these salts are added to a solution of a metal gelatinate only a depressing effect of the Ca or Na ion is apparent but no augmenting effect of the anion.[1] The theoretical reason for these effects will be given in Chap. VIII.

An approximately 1.6 per cent solution of isoelectric gelatin was prepared and brought to a pH of 4.0. The solution was made 0.8 per cent in regard to the originally isoelectric gelatin by adding to 50 c.c. of the 1.6 per cent solution either 50 c.c. of H_2O or of a salt solution, *e.g.*, NaCl, of different molecular concentration, from M/8,192 to 1 M, taking care that the hydrogen ion concentration remained the same. The time of outflow through a viscometer was determined in the way described in Chap. V, and the ratios of the time of outflow to that of water were plotted as ordinates over the pH as abscissæ (lower curve, Fig. 26). We

[1] The contents of this chapter are based on Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 391, 1920–21.

will designate this value as relative viscosity. The addition of the NaCl causes only a drop, and no rise in the curve.

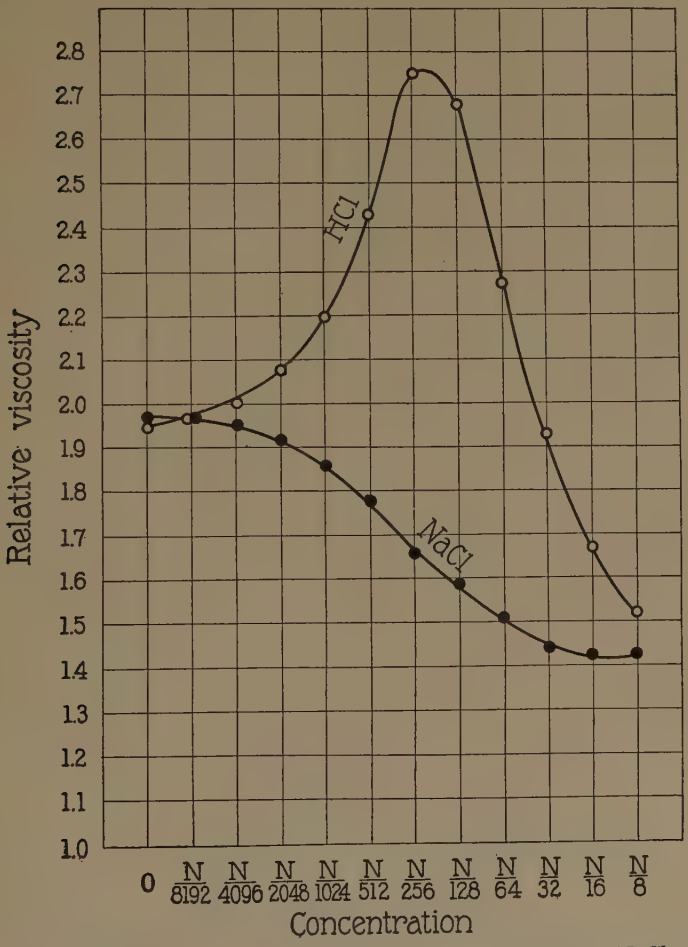

FIG. 26.—Difference in the effect of different concentrations of NaCl and of HCl on the relative viscosity of an 0.8 per cent solution of gelatin chloride of pH 4.0. In the case of NaCl we observe only the depressing effect of the Cl ion; in the case of HCl we notice an augmenting effect of the H ion and a depressing effect of the Cl ion, the latter prevailing as soon as the concentration of acid added is $> N/256$.

If, however, the 1.6 per cent gelatin solution of pH 4.0 is mixed with various concentrations of HCl (upper curve, Fig. 26) instead

of with NaCl, at first a rise occurs which is followed by a drop when the concentration of the Cl ion is a little above N/1,000. In Fig. 26 the drop appears at a concentration of about N/256 HCl, but the reader must remember that on account of the fact that

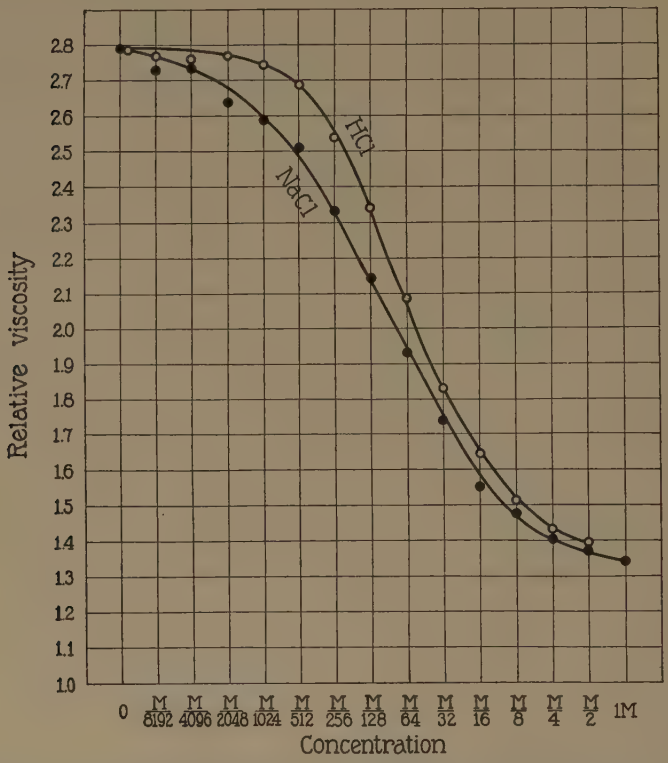

FIG. 27.—The relative viscosity of 0.8 per cent solution of gelatin chloride of pH 3.0 is depressed almost equally by the Cl ion of HCl as of NaCl. The augmenting effect of the H ion in the case of HCl is no longer noticeable.

part of the acid combined with the gelatin the pH of the solution was about 3.0. In other words, while the addition of H ions increases the viscosity of a solution of gelatin chloride of pH 4.0, the addition of Na ions does not have such an effect, but the Cl ion depresses the viscosity in both cases, no matter whether NaCl or HCl is added to the gelatin solution; and the depressing action of the Cl ion increases with its concentration. Moreover,

the increase of the viscosity by the H ions stops as soon as the pH of the solution reaches about 3.0 for the reason stated.

When the same experiment is repeated with a gelatin solution of pH 3.0, the addition of NaCl immediately causes a drop also

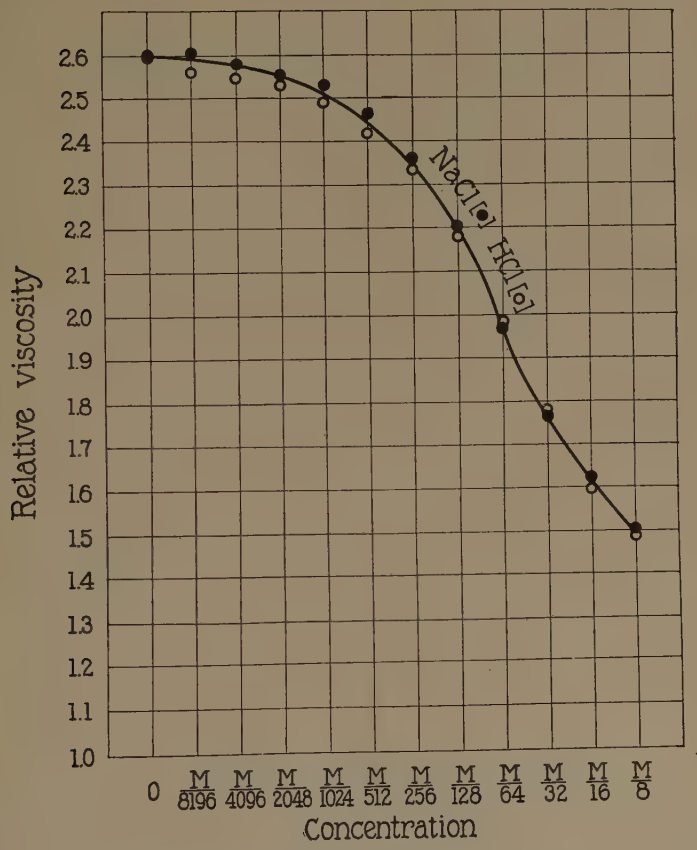

FIG. 28.—When the gelatin solution has a pH of 2.5, HCl and NaCl depress the relative viscosity of the gelatin solution to the same degree.

(Fig. 27) while the addition of HCl no longer causes a rise but the drop commences a little later than in the case of NaCl.

When, however, the same experiment is made with a gelatin solution of pH 2.5 (Fig. 28), an immediate drop is noticed upon the addition of HCl as well as in the case of the addition of NaCl,

and the curve for HCl coincides practically with that for NaCl, as our theory demands.

That the depression of the viscosity of gelatin chloride due to the presence of a salt is exclusively determined by the anion of

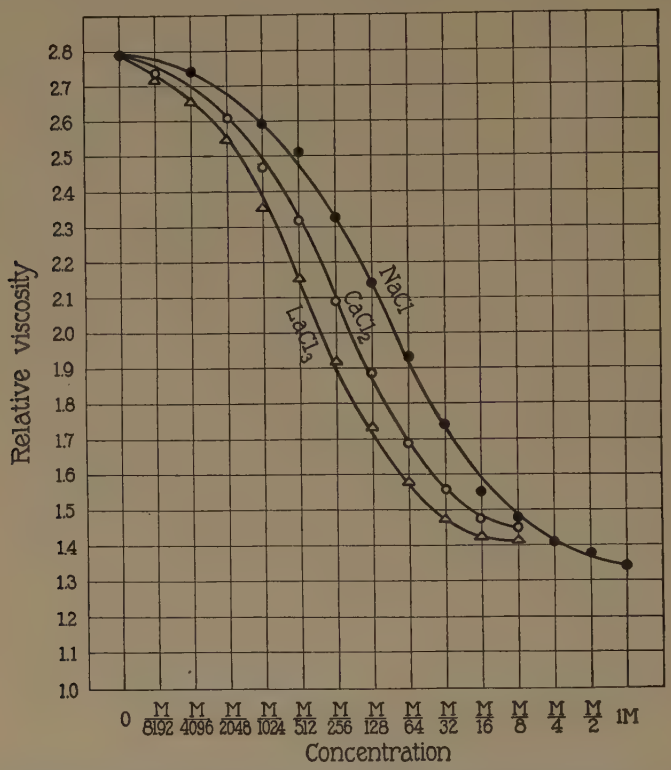

FIG. 29.—The depressing effect of equal molecular concentrations of NaCl, $CaCl_2$, and $LaCl_3$ on the relative viscosity of 0.8 per cent gelatin chloride solution of pH 3.0 is roughly in proportion to the concentration of the Cl ions in the solutions; *i.e.*, as 1:2:3.

the salt and that the cation has no augmenting effect is shown in Fig. 29, where the influence of NaCl, $CaCl_2$, and $LaCl_3$ upon the viscosity of gelatin of pH 3.0 is represented. Fifty cubic centimeters of a 1.6 per cent solution of gelatin chloride of pH 3.0 were added to 50 c.c. of a solution of different concentrations of each salt as described, the pH being kept at 3.0. It is obvious from

Fig. 29 that the molecular concentrations of NaCl, $CaCl_2$, and $LaCl_3$, which depress the viscosity to the same level are approximately in the ratio of 3:2:1. Thus, when the effect of NaCl and

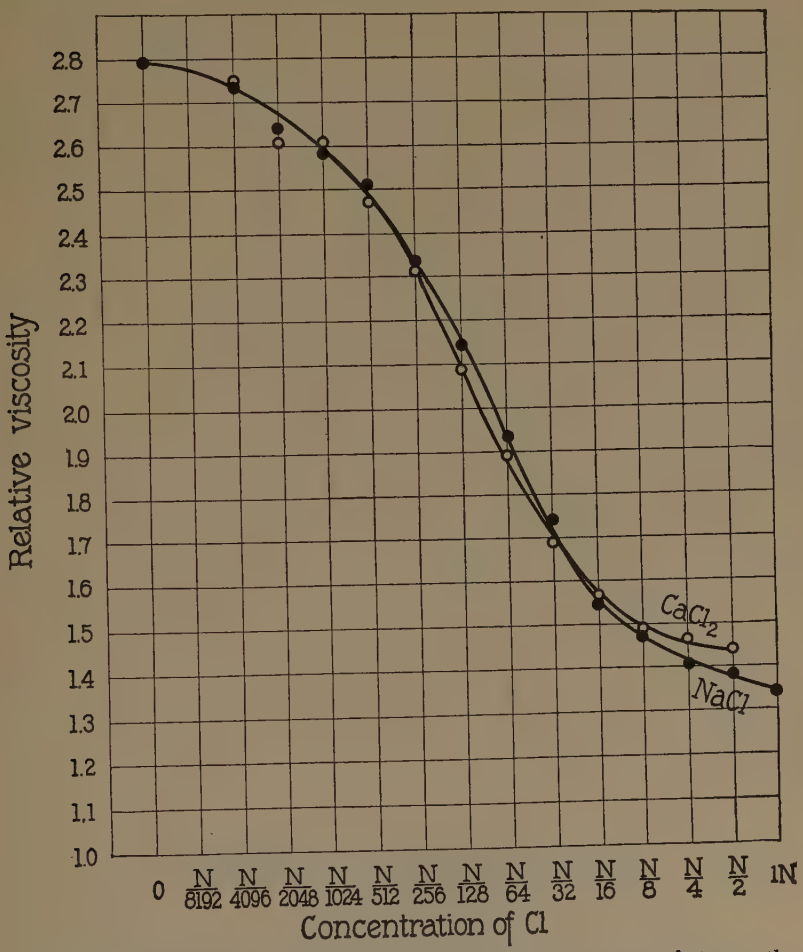

Fig. 30.—Showing that NaCl and $CaCl_2$ have the same depressing effect on the viscosity of gelatin chloride of pH = 3.0 when the concentration of Cl ions is the same.

$CaCl_2$ is plotted over the same concentration of the Cl ions the curves for the salts become nearly identical (Fig. 30), and the same would be practically true for the $LaCl_3$ curve. From this it follows

that the depressing effect of these three salts on gelatin chloride is practically exclusively a function of the

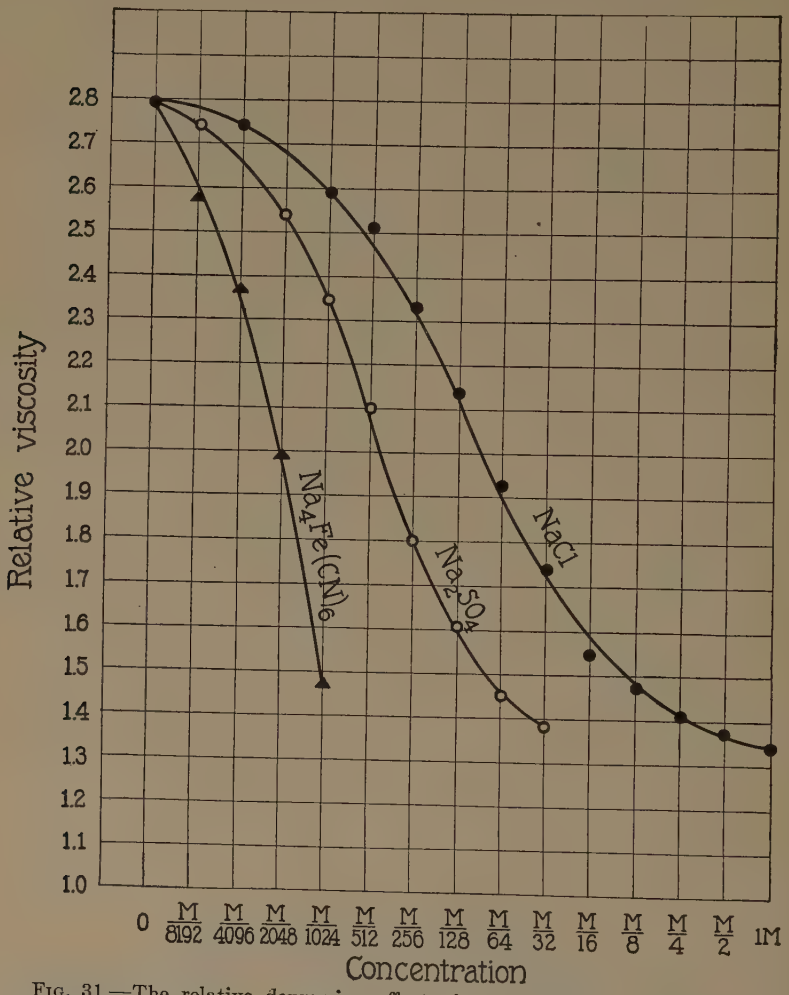

FIG. 31.—The relative depressing effect of equal molecular concentrations of NaCl, Na_2SO_4, and $Na_4Fe(CN)_6$ on the relative viscosity of a gelatin chloride solution of pH 3.0 is approximately as 1:4:16.

Cl ion, while no augmenting effect of the cation is noticeable. This observation disposes of vague hints found in the literature

of colloids that the opposite ions of a neutral salt affect the properties of a protein in an opposite direction. We made sure that in all these cases the pH of the gelatin solution was not altered by the addition of the salt.

When 0.8 per cent solutions of gelatin chloride of pH 3.0 are prepared in solutions of Na salts with the anion of a weaker acid, *e.g.*, Na_2 oxalate, $Na_4Fe(CN)_6$, the pH is increased and there exists the danger of erroneously attributing a depressing effect to the anion which in reality is caused by the increase in pH. In Fig. 31 the effects of the addition of equal concentrations of NaCl, Na_2SO_4, and $Na_4Fe(CN)_6$ on gelatin chloride of pH = 3.0 are plotted. In the case of $Na_4Fe(CN)_6$ only the lowest concentrations, from M/8,192 to M/1,024, could be used, since in these only did the pH of the protein solution remain = 3.0. Figure 31 shows that the depressing effect of these salts increases rapidly with the valency of the anion. When the concentration of the salt was only M/1,024 a drop in the viscosity was already noticeable. This drop was small in the case of NaCl (from 2.8 to 2.6), was greater in the case of Na_2SO_4 (from 2.8 to 2.35), and considerably greater in the case of $Na_4Fe(CN)_6$ (from 2.8 to 1.5). The objection might be raised that since Na_2SO_4 has twice as many cations as NaCl of the same concentration and $Na_4Fe(CN)_6$ has four times as many cations, it was the difference in the concentration of the cations which caused the difference in the drop. This is refuted by the fact that Na_2SO_4 causes a drop in the specific viscosity to 1.8 at a concentration of M/256 while NaCl causes the same drop at a concentration of above M/64 which is about four times as high. If the concentration of the cation were responsible for the drop the two concentrations should be more nearly as 1:2. $Na_4Fe(CN)_6$ causes the same drop of the viscosity to 1.8 at a concentration less than M/1,024. Hence, the concentration of $Na_4Fe(CN)_6$ required to cause the same diminution of the specific viscosity as that caused by M/64 NaCl is less than one-sixteenth of the latter, while it should be only one-fourth if the cation were responsible for the drop.

Experiments on osmotic pressure and on swelling lead to the same formulation of the difference in the effect of acids and salts as the viscosity experiments.

What has been shown for the effect of acids on the physical

properties of proteins can also be shown for the influence of alkalies. Thus, the addition of KOH to Na gelatinate of pH 12.0 depresses the viscosity in the same way as the addition of KCl (Fig. 32); while the addition of little KOH to Na gelatinate of pH 4.8 to 8.0 increases the viscosity, and the addition of KCl to Na gelatinate always depresses the viscosity. The depressing effect of salts on the viscosity of solutions of metal gelatinate is

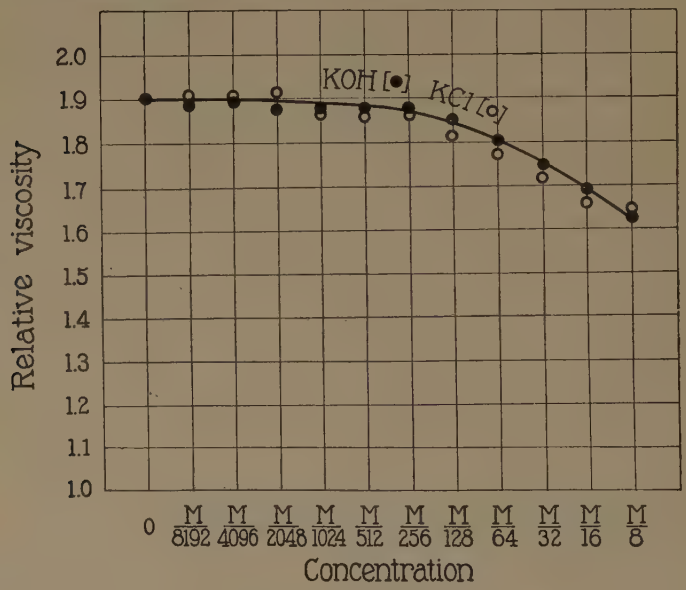

FIG. 32.—The depressing effect of KOH and KCl on Na gelatinate of pH 12.0 is practically the same.

due to the cation of the salt added, that of bivalent cations being greater than that of monovalent cations, while the valency of the anion has no effect.

We have already stated that the addition of neutral salt to isoelectric gelatin leaves the viscosity and osmotic pressure of the solution practically unchanged. This fact is of great importance for the theory of colloids.

The depressing effect of neutral salts on the physical properties of proteins is, therefore, the same phenomenon as the drop in the curves of these properties when too much acid or too much alkali has been added. It is due to the fact that in all

cases that ion which has the opposite sign of charge to that of the protein ion depresses the osmotic pressure, swelling, and viscosity of proteins.

2. Ion Series and the Action of Salts on Proteins

From what has been said, it is clear that only one of the ions of a neutral salt influences the physical properties of a protein, namely that ion which has the opposite sign of charge to the protein ion; and this influence is of a depressing character. We will now show that this effect depends only upon the valency of the depressing ion and that different ions of the same valency have the same depressing effect. It is necessary to compare the relative depressing action of low but equal concentrations of different salts upon the physical properties of a gelatin salt, for example, gelatin chloride of a definite pH; *e.g.*, 3.0. As can be easily surmised, the addition of a salt will in many cases alter the pH of the solution and this alteration will be larger in the case of certain salts, *e.g.*, Na acetate, than in the case of others, *e.g.*, NaCl. Unless we take into consideration these variations in the pH caused by the addition of salts there will be danger of erroneously ascribing the influence of a variation in the hydrogen ion concentration to an influence of the nature of the anion. The Hofmeister ion series, as far as they refer to proteins, are largely due to this error.

The method of our experiments was as follows: 50 c.c. of a 1.6 per cent solution of originally isoelectric gelatin contained enough HCl to make the pH = 3.0. To this were added 50 c.c. of H_2O or of a salt solution of different molecular concentration, and the viscosity of this mixture was measured using those precautions which were described in the preceding chapter.

Figure 33 gives the curves representing the depression of the relative viscosity of a gelatin chloride solution of pH 3.0 by different concentrations of salts with monovalent anion; namely, NaCl, NaH_2PO_4, NaCNS, NaH tartrate, NaH_2 citrate, and Na acetate. The curve for Na_2SO_4 is added for comparison. The monosodium salts of weak dibasic and tribasic acids dissociate electrolytically into a Na ion and a monovalent anion, H_2PO_4, H tartrate, H_2 citrate, etc. All the salts mentioned in Fig. 33 are therefore salts with monovalent anion with the exception of

Na_2SO_4. Our valency rule demands that the relative depressing

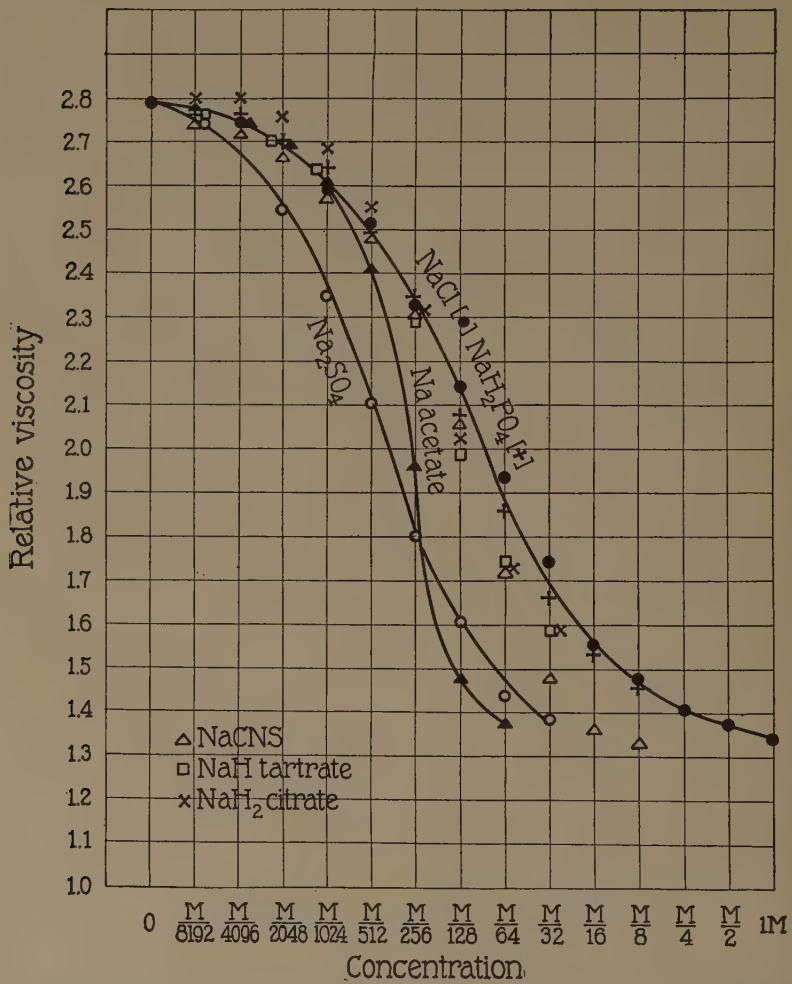

FIG. 33.—The depressing effect of different salts with monovalent anion (NaCl, NaH_2PO_4, NaCNS, NaH tartrate, and NaH_2 citrate) on the relative viscosity of 0.8 per cent solution of gelatin chloride of pH 3.0. The effects of NaCl and NaH_2PO_4 are identical since the pH is not altered by the addition of these salts. The depression in the values for the relative viscosity is greater in the case of Na acetate than in the case of NaCl for the reason that the Na acetate raises the pH of the gelatin chloride solution.

effect of these salts (with the exception of Na_2SO_4) should be

nearly the same and that deviations from this rule should find their explanation in corresponding deviations of the pH due to the influence of certain of the salts. We will first consider this latter influence as given in Table VI, which shows the results of the

TABLE VI.—CHANGES IN pH OF 0.8 PER CENT GELATIN CHLORIDE OF pH = 3.0 UPON ADDITION OF VARIOUS CONCENTRATIONS OF SALTS

	Molecular concentrations of salts used												
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
NaCl	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Na_2SO_4	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.05	3.1	3.2	3.3	3.35
NaH_2PO_4	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.1	3.2	3.3	3.4	3.45	3.5
NaCNS	3.0	3.0	3.0	3.0	3.0	3.0	3.1	3.2	3.3	3.6	3.9	4.2	4.4
NaH tartrate	3.0	3.0	3.0	3.0	3.0	3.0	3.1	3.3	3.45	3.5	3.55		
NaH_2 citrate	3.0	3.0	3.0	3.0	3.0	3.1	3.2	3.4	3.6	3.7	3.75		
Na acetate	3.0	3.0	3.0	3.05	3.1	3.3	3.7	4.3	4.6				

measurements of pH in these different gelatin solutions after the addition of salts. The original gelatin chloride solution had a pH of about 3.0 and the pH was not altered by the addition of NaCl and only slightly by the addition of NaH_2PO_4 in concentrations below M/16. According to the valency rule the curves for the depressing effect of NaCl and NaH_2PO_4 should be almost identical and Fig. 33 shows that this is the case.

Table VI shows that NaCNS, monosodium tartrate, and monosodium citrate raise the pH of the solution as soon as the concentration reaches M/128 or more. If we consider this effect, we must expect to find that the drop in the curves for NaCNS, monosodium citrate, and monosodium tartrate is a little steeper in concentrations of M/128 and above than the curve for the depressing effect of NaCl. Figure 33 shows that the curves for the depressing effect of these three salts are slightly lower than the curve for NaCl or NaH_2PO_4. The greatest apparent deviation from the valency rule occurs in the curve for Na acetate whose depressing effect is of the order of that of Na_2SO_4.

In the colloidal literature it is always stated that Na acetate acts like Na_2SO_4 and this is interpreted to mean that the acetate

anion acts like the bivalent SO_4 anion and not like the monovalent Cl or NO_3 anion. Table VI shows that Na acetate also depresses the hydrogen ion concentration more than NaCl or NaH_2PO_4;

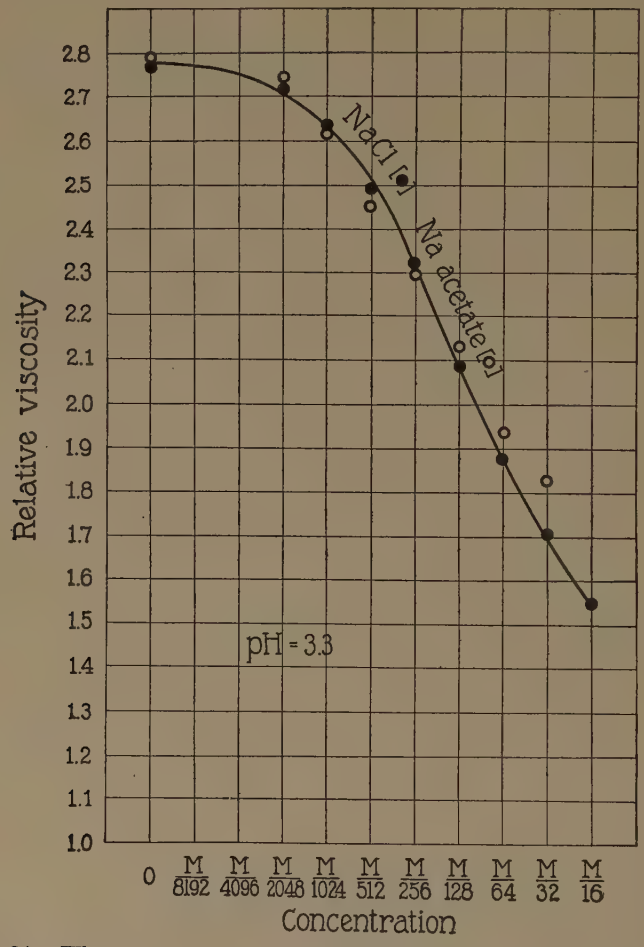

FIG. 34.—When the pH is kept equal the depressing effect of equal concentrations of NaCl and Na acetate on the relative viscosity of an 0.8 per cent gelatin chloride or gelatin acetate solution of pH 3.3 is the same.

M/64 Na acetate brings the gelatin solution practically to the isoelectric point, and at the isoelectric point the viscosity of gelatin solution is a minimum. This lowering of the hydrogen ion

concentration (and not the alleged influence of the acetate anion) explains the excessive depressing effect of Na acetate. That this interpretation is correct can be proved in the following way: 0.8 per cent solutions of gelatin acetate of pH 3.3 and gelatin chloride also of pH 3.3 were prepared. The relative viscosity of these two solutions was practically the same (both were 0.8 per cent solutions in regard to originally isoelectric gelatin). The solution of gelatin acetate of pH 3.3 was made up in various concentrations of Na acetate of pH 3.3. The Na acetate solution of pH 3.3 was obtained by dissolving M/16 Na acetate in 1½ M acetic acid and the various degrees of dilution of this M/16 Na acetate solution of pH 3.3 were brought about by dilution with pure acetic acid of pH 3.3. The non-dissociated molecules of acetic acid have no more depressing influence on the physical properties of proteins than have the molecules of any non-electrolyte. Figure 34 gives the curve representing the depressing effect of Na acetate on gelatin acetate of pH 3.3, when the pH is kept constant.

The gelatin chloride solution of pH 3.3 was made up in different concentrations of NaCl and the depressing effect of NaCl on the viscosity of gelatin chloride is also plotted in Fig. 34. It is obvious from Fig. 34 that the depressing effects of Na acetate and NaCl are identical when the pH is kept constant and identical in both cases.

The same fact was confirmed in a somewhat different way. A 1.6 per cent solution of gelatin chloride of pH 3.0 was made up in various concentrations of Na acetate also of pH 3.0. In order to prepare Na acetate solutions of pH 3.0, M/4 Na acetate was dissolved in M/4 HCl and the various dilutions required for the experiment were obtained by diluting the mixture of equal parts of M/4 HCl and M/4 Na acetate with M/1,000 HCl.

The 1.6 per cent gelatin chloride solution of pH 3.0 was diluted with 50 c.c. of this mixture so that the resulting 0.8 per cent gelatin chloride solution of pH 3.0 contained various concentrations of Na acetate (or more correctly of NaCl and Na acetate). The curve representing the depressing effect of this salt is given in Fig. 35, and is shown to be identical with the curve representing the depressing effect of the addition of NaCl to gelatin chloride of pH 3.0.

We can, therefore, state that sodium acetate has the same effect on the viscosity of gelatin chloride as the addition of any other salt with monovalent anion, and that the anomalous effect

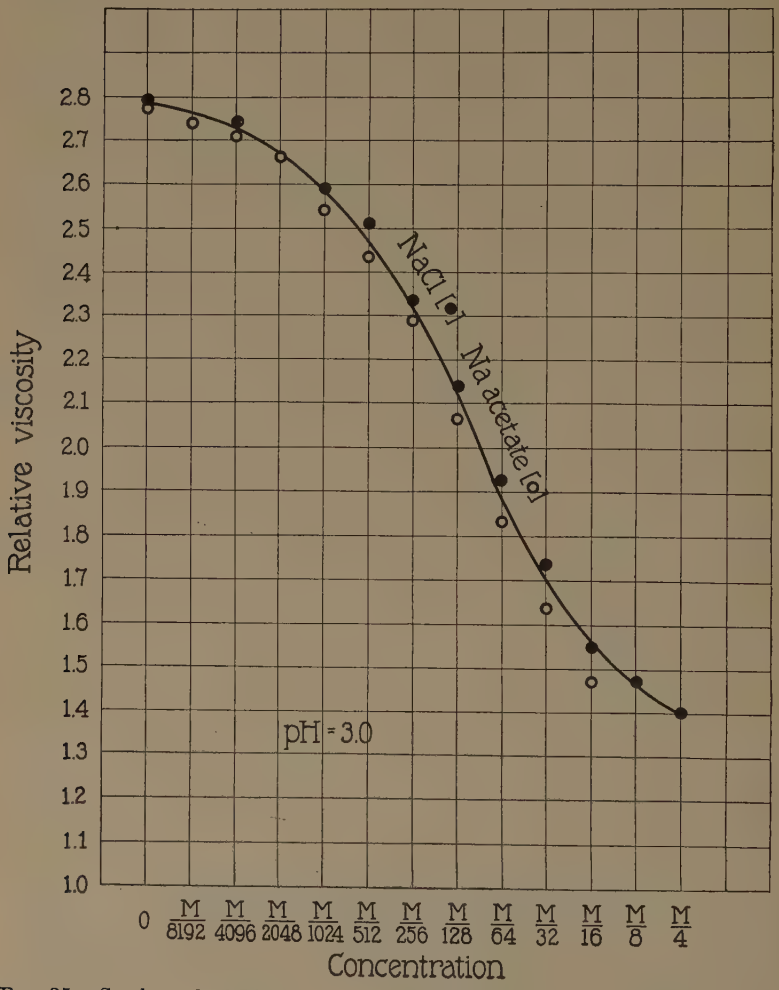

FIG. 35.—See legend of Fig. 34, except that the pH of gelatin solution is 3.0.

ascribed to the acetate anion in the colloidal literature is in reality due to the depression of the hydrogen ion concentration of the gelatin solution by the Na acetate, which is a buffer salt.

The failure to recognize the buffer character of salts, like the acetates, citrates, and tartrates, has led to the error of the Hofmeister ion series. In reality we find our valency rule confirmed whereby all salts with an anion of the same valency have about the same relative depressing effect on the viscosity of a gelatin chloride solution if the pH of the solution is kept constant.

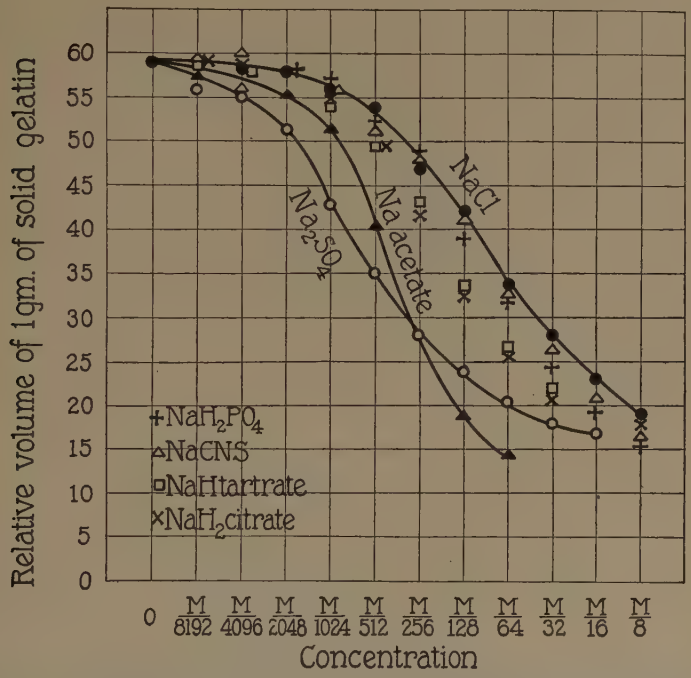

FIG. 36.—Showing that the depressing effect of salts with monovalent anion on the swelling of gelatin chloride of pH 3.3 is similar to that on the relative viscosity. All salts with monovalent anion depress the swelling of gelatin chloride to the same extent, the seeming deviation from this rule being due to variation in the pH of the gelatin solution caused by buffer salts.

What has been demonstrated for the effect of these salts on the viscosity of gelatin solutions holds also for their effect on the swelling of gelatin. The same volumetric method for measuring the swelling effect was used which was described in the preceding chapter. Figure 36 gives the relative depressing effect of NaCl, NaH_2PO_4, NaCNS, monosodium tartrate, monosodium citrate,

and Na acetate on the swelling of gelatin chloride of pH 3.3 (the curve for Na_2SO_4 is added for comparison), and Table VII gives the variation of the pH of the gelatin caused by the addition of these salts. Our theory demands that all these salts (except Na_2SO_4) should depress the swelling of gelatin chloride of pH 3.3 to the same amount, and that deviations from this rule

TABLE VII.—CHANGES IN pH OF 0.8 PER CENT GELATIN CHLORIDE OF pH = 3.3 UPON ADDITION OF VARIOUS CONCENTRATIONS OF SALTS

	Molecular concentrations of salts used											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512.	M/256	M/128	M/64	M/32	M/16	M/8
NaCl	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Na_2SO_4	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.35	3.4	3.5	3.6
NaH_2PO_4	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.4	3.5	3.6	3.7
NaCNS	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.35	3.4
NaH tartrate	3.3	3.3	3.3	3.3	3.3	3.4	3.5	3.5	3.6	3.7	3.7	3.7
NaH_2 citrate	3.3	3.3	3.3	3.3	3.3	3.4	3.5	3.6	3.8	3.85	3.9	3.9
Na acetate	3.3	3.3	3.3	3.4	3.45	3.5	3.8	4.3	4.8	5.2	5.4	5.5

must find their explanation in variations of pH caused by the addition of salt. Table VII shows that the variations in pH are small for NaCl, NaCNS, and NaH_2PO_4 and hence, the curves for the depressing effect of these three salts upon the swelling of gelatin are almost identical, as the valency rule demands. Monosodium citrate and tartrate have a greater depressing effect on the hydrogen ion concentration and Na acetate has a still greater depressing effect than these two salts. This explains the apparent deviation of the curves for these three salts from the valency rule.

A. D. Hirschfelder[1] has published a paper on the effects of different salts on the swelling of fibrin in HCl in which he showed that the effect of citrates, acetates, and phosphates on swelling was the same as that of chlorides, bromides, and nitrates if the hydrogen ion concentration was kept constant; only the sulphates had a greater depressing effect. The influence of salts on the

[1] Hirschfelder, A. D., *J. Am. Med. Ass.*, vol. 67, p. 1891, 1916.

swelling of fibrin is, therefore, identical with the influence of salts on the swelling of gelatin.

The osmotic pressure, viscosity, and swelling of Na gelatinate should be depressed by the cation of a salt and the more so the higher the valency of the cation. Figure 37 shows that this is true for the swelling of Na gelatinate of pH about 9.3. The molecular concentration in which the swelling is depressed by the

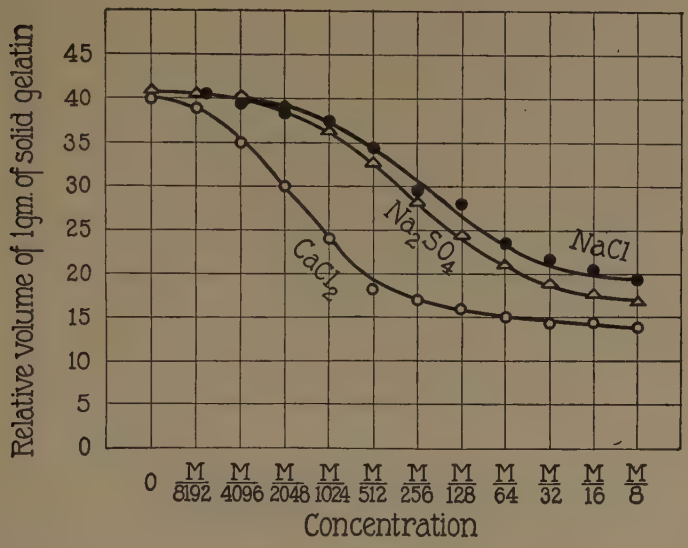

FIG. 37.—The depressing effect of neutral salts on the swelling of Na gelatinate of pH about 9.3 is due to the cation of the salt, the depressing effect of NaCl being half as great as that of Na_2SO_4 of equal molecular concentration of Na_2SO_4 while that of $CaCl_2$ is considerably greater owing to the fact that Ca is bivalent.

same amount is about half as great for Na_2SO_4 as for NaCl (for molecular concentrations from M/256 to M/32), while it is about eight times as high for NaCl as for $CaCl_2$, roughly proving that the cation is responsible for the depression. The pH of the gelatin was practically the same in all solutions.

All these data confirm our valency rule, whereby ions of the same valency and the same sign of charge have, in the same concentration, nearly the same depressing effect on osmotic pressure, swelling, and viscosity of proteins; while the depressing effect increases rapidly with the valency. The Hofmeister ion

series are chiefly due to the failure to measure the influence of the salts on the hydrogen ion concentration of the gelatin solutions. This neglect has given rise to the statement that salts like sodium acetate have the same depressing effect on the physical properties of proteins as the sulphates.

Neutral salts, when added in low concentrations—*i.e.*, below M/16—affect the physical properties of proteins in two different ways: first, by an exchange of one of the ions of the salt for the ion with which the protein is in combination. Thus by adding K_2SO_4 to a solution of gelatin chloride, gelatin sulphate is formed resulting in a diminution of osmotic pressure, viscosity, etc., of the protein solution; or if KCl is added to gelatin sulphate the reverse chemical and physical changes take place. If the protein is on the alkaline side of its isoelectric point, *e.g.*, in the case of Na gelatinate, the addition of a salt with bivalent cation, *e.g.*, $MgCl_2$ or $CaCl_2$, etc., results in the formation of Mg or Ca gelatinate with the consequence that the osmotic pressure, viscosity, and swelling of the gelatin is diminished. By mixing two different salts, *e.g.*, NaCl and $MgCl_2$, the antagonistic effects so well known in biology can be imitated.

The second effect of the addition of a neutral salt to a solution of a protein is a general depressing effect on the physical properties of a solution of a protein salt and this depression is caused by that ion of the salt which has the opposite sign of charge to that of the protein ion. Thus all anions regardless of valency depress the osmotic pressure, viscosity, and swelling of gelatin chloride and the depressing effect increases with the concentration and valency of the anion of the salt added. All cations depress the viscosity, swelling, and osmotic pressure of Na gelatinate and the more so the higher the concentration and valency of the cations added.

This effect is similar to the depression of electrolytic dissociation of one electrolyte caused by the addition of a second electrolyte with a common ion, but, nevertheless, the salt effects just mentioned are not (or only to a negligible degree) due to a depression of the degree of electrolytic dissociation of the protein salt, but are due to Donnan's membrane equilibrium.

Previous authors had already observed that only electrolytes have a depressing effect on the physical properties of protein

solutions, such as osmotic pressure, viscosity, etc., while non-electrolytes, like cane sugar, have no such effect. Since in these older experiments the pH was not considered and since this fact is of paramount importance, it seemed desirable to repeat them. It was found that non-electrolytes, like cane sugar, have no depressing effect on the osmotic pressure or the viscosity of gelatin solutions. Solutions of gelatin chloride of pH 3.4 containing 1 gm. of originally isoelectric gelatin in 100 cc. solution were made up in various concentrations of cane sugar, were rapidly heated to 45° and rapidly cooled to 24°. The time of outflow of the gelatin solutions through a viscometer was measured immediately. In addition the time of outflow of the pure sugar solution was also determined at 24°C. The ratio of the time of outflow of the gelatin-cane sugar solution divided by the time of outflow of the pure cane sugar solution was thus determined. The results given in Table IX show that the ratio of viscosity of gelatin solution to viscosity of cane sugar solution is not diminished by the addition of cane sugar; in fact it seems, if anything, slightly increased if the cane sugar concentration is above M/8.

TABLE IX.—INFLUENCE OF THE ADDITION OF CANE SUGAR ON THE VISCOSITY AND OSMOTIC PRESSURE OF 1 PER CENT SOLUTIONS OF GELATIN CHLORIDE OF PH 3.4

	Concentration of cane sugar										
	0	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2
Viscosity ratio.....	2.33	2.31	2.33	2.35	2.30	2.31	2.31	2.30	2.39	2.44	2.57
Osmotic pressure after 21 hours at 24°C. in millimeters H_2O....	434	390	380	405	408	400	407	432	397	401	395

Similar results were obtained in regard to osmotic pressure as Table IX shows.

This fact is one of the prerequisites for the validity of the theory of membrane equilibrium, since only ions contribute to the equilibrium conditions on opposite sides of the membrane.

A second prerequisite is, that the addition of salts should have

no influence on the viscosity, osmotic pressure, or P.D. of protein solutions at the isoelectric point. This prerequisite of the Donnan theory was also fulfilled.

In his book on "Applied Colloid Chemistry" Bancroft makes the following comment on the writer's experiments on the Hofmeister series.

"Under the conditions of the experiments Loeb found that on the acid side of the isoelectric point only anions of neutral salts are taken up and on the alkaline side of the isoelectric point only cations. Since the Hofmeister series calls for an effect due to both ions of a neutral salt on the swelling of gelatine, Loeb concludes that the Hofmeister series is a delusion and a snare. This does not follow at all. Loeb is working at such extreme dilutions that the specific effects of all ions but hydrogen and hydroxyl ions are practically negligible. In acid solutions only anions are taken up and in alkaline solutions only cations. Loeb recognizes the specific effect of iodine ions over chlorine ions in causing the liquefaction of gelatine; but he considers that liquefaction stands in no necessary relation to swelling, an assumption which will be shared by few. With higher salt concentrations Loeb will undoubtedly get entirely different results."[1]

The answer to this comment is that when Bancroft wrote it he had not read the writer's later papers dealing with the Hofmeister series. A glance at Figs. 27, 29, 30, 31, 33, 35, 46, and 60 will show that salt solutions up to grammolecular concentration were used without any indication of the validity of the Hofmeister series being found. Bancroft will surely not maintain that solutions of neutral salts up to molecular concentration are so dilute that the effects of all ions except the hydrogen and hydroxyl ions are practically negligible.

The writer's statement that the liquefaction of solid gelatin stands in no necessary relation to swelling is correct, since higher concentrations of acids or of salts like $CaCl_2$ diminish the swelling of gelatin while they increase its solubility (see Chap. XIV). This is due to the fact that swelling and solution of solid gelatin in the presence of acid are functions of different variables, swelling in acid depending on the Donnan equilibrium, while the solution of gelatin depends on the same forces which are responsible for the solution of ordinary crystalloids in water (probably secondary valency forces).

The belief in the validity of the Hofmeister series has given rise

[1] Bancroft, W. D., "Applied Colloid Chemistry," New York and London, 1921, pp. 255–256.

to a flood of speculations concerning the nature of physiological and pathological processes. These speculations were, unfortunately, rarely supported by adequate experimntes and when experiments were made the hydrogen ion concentrations were ignored, so that the basis of these speculations is always uncertain if not positively wrong. Thus it has been suggested that muscular contraction is due to swelling caused by acid formation. This may or may not turn out to be correct, but the production of acid in the muscle can only lead to increased swelling if the pH inside the muscle is lower (but not much lower) than that of the isoelectric point of the proteins responsible for the alleged swelling; since otherwise the acid formation could only diminish the swelling already existing in the resting muscle. It is obvious that we must know the isoelectric points of the proteins in the muscle, as well as the pH in the resting and the active muscle, before a discussion of the hypothesis becomes profitable.

It has been stated that edema is due to the swelling of proteins inside the cells caused by acid formation. Not only have none of the measurements of the hydrogen ion concentrations required for such a hypothesis been made but all critical experiments and clinical observations indicate that edema is a phenomenon dependent on increased filtration of liquid from the capillaries into the spaces between tissues or cells; while there is no indication that edema is connected with colloidal swelling.[1]

It has been suggested that the absorption of water by the striped muscle (and by other cells) in hypotonic solutions is due to a colloidal swelling caused by acid formation inside the cells, but it can be shown that if the solution is rendered isotonic by the addition of a sugar, the living muscle no longer absorbs water.[2] This proves that the absorption of water by living muscles (and other living cells) in hypotonic solution is due to the fact that these tissues or cells are surrounded by semipermeable membranes and that the absorption of water by living striped muscles or cells in hypotonic solutions has no connection with colloidal swelling.

[1] See HIRSCHFELDER, A. D., *Trans. Section Pharmacol. and Therapeutics*, Am. Med. Assoc., p. 182, 1917; and MOORE, A. R., *Am. J. Physiol.*, vol. 37, p. 220, 1915.

[2] HÖBER, R., "Physikalische Chemie der Zelle und der Gewebe," p. 386, Leipsic and Berlin, 1914. LOEB, J., *Science*, vol. 37, p. 427, 1913.

CHAPTER VII

THE INADEQUACY OF THE PRESENT THEORIES OF COLLOIDAL BEHAVIOR

We have given a survey of the influence of electrolytes on the behavior of proteins and we may now single out those characteristics which are specifically colloidal, *i.e.*, which do not seem to occur in crystalloids. These characteristics are:

1. The addition of little acid (or alkali) to an isoelectric protein (crystalline egg albumin, gelatin, and casein) increases, and the addition of more acid (or alkali) diminishes the osmotic pressure, the viscosity (and also, as will be seen, the potential differences) of solutions of these proteins; and the same is true for the swelling of gelatin.

2. This effect of acids and alkalies depends only on the sign and the valency of the ions in combination with the proteins; ions of the same sign and valency, *e.g.*, Cl, NO_3, CH_3COO, H_2PO_4 HC_2O_4 etc., influence the properties in the same way, provided that th,e properties of the protein solutions are compared for the same pH and the same concentration of originally isoelectric protein, and provided that no constitutional changes occur in the protein molecule or ion.

3. When the ion in combination with a protein is bivalent (*e.g.*, SO_4, Ca, Ba) the osmotic pressure, viscosity, and swelling of the protein are considerably less than when the ion is monovalent (*e.g.*, Cl, Br, NO_3, H_2PO_4, HC_2O_4, Na, K, etc.).

4. The addition of a neutral salt to a protein solution (which is not at the isoelectric point) depresses the osmotic pressure, viscosity (and P.D.) of the solutions and the degree of swelling of gels, and this effect increases with the valency of that ion of the salt which has the opposite sign of charge to that of the protein ion.

Any theory which claims to be able to explain colloidal behavior must account quantitatively for these four results. As a matter

of fact the explanations offered in the colloidal literature do not even suffice as qualitative explanations since they are in contradiction with the facts.

We have seen in the introduction how the original definition of colloids by Graham, based on the non-diffusion of colloids (through membranes), has of late been abandoned by colloid chemists in favor of the micella or aggregation theory of colloids, according to which the ultimate unit of colloidal matter in solution or suspension is not the isolated molecule or ion, but an aggregate of the latter—the micella of Naegeli. Such aggregations occur and they play a role in gel formation, precipitation, and to some extent in the viscosity of protein solutions, but they cannot explain the influence of electrolytes on the properties of proteins mentioned, since they have only an indirect connection with colloidal behavior. It will be shown that the aggregates act like membranes blocking the diffusion of the ions constituting the aggregate and this prevention of diffusion is a source of colloidal behavior.

The depressing effect of the addition of salts to protein solutions cannot be harmonized with the aggregation theory. Zsigmondy suggests that the depressing effect of a neutral salt on the osmotic pressure of a solution of a gelatin salt might find its explanation in the assumption that the addition of salt increases the degree of aggregation and hence diminishes the number of the particles in solution, the diminution in the number of particles leading to the lowering of osmotic pressure.[1] It is undoubtedly true that salts precipitate proteins and that precipitation is due to an increase in aggregation, but the salting out of gelatin from its watery solution is not determined by the ion with the opposite sign of charge to that of the protein ion, while we have seen that the depressing effect of a salt on the osmotic pressure of gelatin solutions is determined by the ion with the opposite sign of charge to that of the protein ion. In other words, the salting out of gelatin from its watery solution is a process of an entirely different character from the lowering of the osmotic pressure of a protein solution by a neutral salt. It is, therefore, impossible to explain the latter process by the former.

[1] ZSIGMONDY, R., "Kolloidchemie," 2nd ed., p. 342, Leipsic, 1918.

Moreover, the attempt to explain the depressing effect of the addition of salts on the basis of the micella theory fails completely in the case of the other properties of protein solutions which are equally depressed by them as the osmotic pressure, namely, the viscosity and the P.D.

We shall see in Chap. XIII that if the state of aggregation increases in a gelatin solution—*i.e.*, if isolated protein molecules or ions unite to form a larger aggregate—the viscosity of the solution is thereby increased, for the reason that these aggregates occlude comparatively large quantities of water whereby the relative volume occupied by the gelatin in the solution is increased. This increase in the volume of the micellæ at the expense of water leads, as will be seen, to an increase in viscosity. Hence, if we assume that the addition of a salt increases the degree of aggregation in protein solution, it would follow that this should result in an increase of viscosity; while the addition of salt depresses the viscosity. The attempt to explain the depressing influence of salts on the osmotic pressure and viscosity of protein solutions on the basis of the aggregation theory leads therefore to conclusions which are in contradiction with the actual facts.

An attempt to account for the colloidal behavior of protein solutions was made by Pauli in his theory of hydration of protein ions.

Kohlrausch had tried to account for the differences in the mobility of different ions by the assumption that each ion is surrounded by a shell of water and that the velocity of migration of ions is greatest where this shell of water is a minimum. Pauli assumes that the protein ion is surrounded by an enormous shell of water while no such jacket of water surrounds the non-ionized protein.[1] The shell of water might prevent the coalescence of the protein ions and, hence, might cause a higher degree of dispersion. On the basis of the hydration theory we can find a qualitative explanation of the peculiar pH curves in the following way: At the isoelectric point protein is in a non-ionized condition and no hydration occurs. Hence, the degree of dispersion of particles and the osmotic pressure are a minimum at this point and the viscosity and swelling should also be a minimum,

[1] Pauli, W., *Fortschr. naturwiss. Forschung*, vol. 4, p. 223, 1912. "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

since swelling might be directly due to the existence of this water jacket, and viscosity should also increase with the mass of water surrounding each particle. If an acid, *e.g.*, HCl, is added to isoelectric gelatin, the latter will be transformed into gelatin chloride which, being a salt, is strongly dissociated. The more acid is added the more gelatin is transformed into gelatin chloride. We have shown in Chap. V that the curves for osmotic pressure, swelling, and viscosity reach a maximum at a pH varying between 3.5 and 2.8, and that they then drop. Pauli assumes that the drop is due to a repression of the degree of electrolytic dissociation of the gelatin chloride (or any protein-acid salt) through the addition of more acid on account of the common anion. It should, however, be mentioned that Pauli[1] and Manabe and Matula[2] state that the maximum of the curves occurs not at pH between 3.5 or pH 2.8, but at pH 2.1 or 2.0.

Table X shows that the pH for the maximal values of the physical properties of gelatin, crystalline egg albumin, and casein solutions is considerably higher than 2.1. In fact at a pH of 2.1 the osmotic pressure of gelatin chloride and albumin chloride solutions is half way down between that at the maximum (pH 3.4) and at the minimum (the isoelectric point, pH 4.7).

TABLE X

1 per cent protein chloride solution	pH where the maximal values are observed for		
	Osmotic pressure	Swelling	Viscosity
Gelatin	3.4	3.2	2.9
Crystalline egg albumin	3.4		
Casein	3.0	...	3.0

The assumption that the maximum lies at pH 2.1, therefore, does not agree with the observations made on the physical behavior of the three proteins mentioned in Table X. It might be true for the viscosity of solutions of blood albumin, on which

[1] PAULI, W., "Kolloidchemie der Eiweisskörper," Dresden and Leipsic, 1920.

[2] MANABE, K. and MATULA, J., *Biochem. Z.*, vol. 53, p. 369, 1913.

Pauli has done most of his work, but Michaelis and Mostynski[1] have pointed out that there is no maximum of viscosity in the case of serum albumin. There is also no maximum in viscosity followed by a drop in the case of egg albumin when the pH varies.

The hydration hypothesis can be put to a direct test by determining the specific conductivity of solutions of protein salts, *e.g.*, gelatin chloride, albumin chloride, etc. Since according to the hydration hypothesis only the protein ion undergoes hydration, the variation in the osmotic pressure, swelling, and viscosity should be accompanied by a corresponding variation in the concentration of protein ions in solution. If, therefore, the specific conductivity of gelatin chloride is measured at varying pH but equal concentrations of originally isoelectric gelatin, the curves representing the values found for conductivity of the protein should run parallel with the curves for the osmotic pressure, swelling, and viscosity; moreover, the curve for the conductivity of gelatin sulphate should be only about half as high as the curve for the specific conductivity of gelatin chloride; while the curve for the specific conductivity of gelatin oxalate should be almost but not quite as high as that for gelatin chloride. The experiments show that this is not the case.

The concentration of ionized gelatin in solution can be determined with the aid of conductivity measurements of the solution of a gelatin salt, *e.g.*, gelatin chloride, by deducting the conductivity of the free HCl in the solution from the total conductivity of the gelatin solution, since the gelatin chloride solution prepared by the writer's method from washed powdered isoelectric gelatin contains practically no other electrolyte except the free HCl and the gelatin chloride. This was proved by ash determinations and by the fact that a solution of isoelectric gelatin prepared according to our method of washing has practically a conductivity of zero. The method of procedure was as follows:

Solutions of different gelatin-acid salts were prepared in two different concentrations of originally isoelectric gelatin, 0.8 per cent and 2.4 per cent. The specific conductivities of these gelatin-acid salts were determined at different pH. The conductivities of pure aqueous solutions of the same acids at different pH were also measured. In both cases the conductivities were plotted

[1] Michaelis, L. and Mostynski, B., *Biochem. Z.*, vol. 25, p. 401, 1910.

as ordinates over the pH as abscissæ. By deducting the values for the specific conductivity of the pure aqueous solution of an acid from the values for the total specific conductivity of the gelatin-acid solution of the same pH the curve for the specific conductivity of the gelatin-acid salt for that pH is obtained.[1]

Figure 38 shows that the curves representing the percentage of ionized gelatin in gelatin chloride resemble the combination

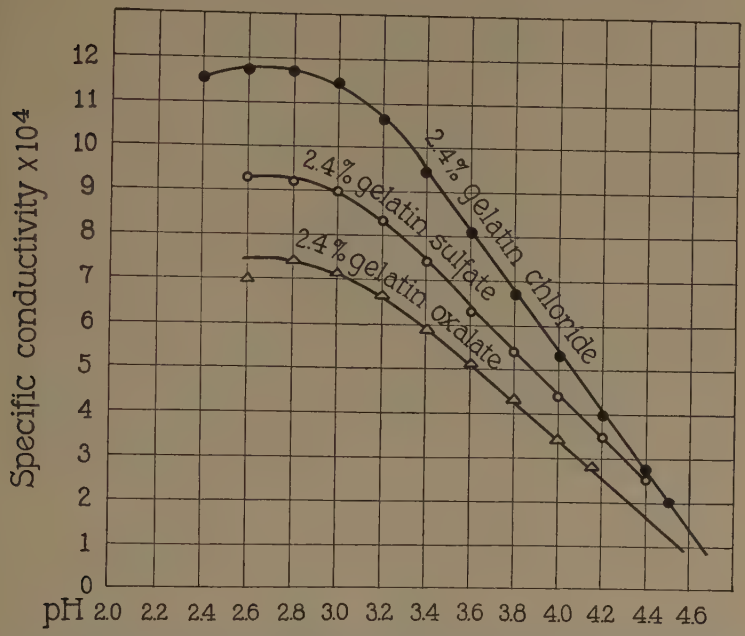

FIG. 38.—Curves for the specific conductivity of 2.4 per cent solutions of gelatin chloride, sulphate, and oxalate, showing the entirely different character of these curves from that of the osmotic pressure curves in Figs. 14 and 15.

curves in Fig. 8, since in both cases there is a gradual rise in the concentration of ionizable protein at a pH below that of the isoelectric point, but no maximum followed by a drop at pH 3.4 or 3.0. But otherwise the curves for combination and for conductivity differ; the curve representing the percentage of ionized gelatin is almost the same for gelatin chloride and gelatin sulphate, while for gelatin oxalate the curve is a little lower. If we attempt

[1] LOEB, J., *J. Gen. Physiol.*, vol. 3, p. 247, 1920–21.

to account for the low osmotic pressure of gelatin sulphate solutions by the hydration hypothesis, the specific conductivity of gelatin sulphate should be half or less than half of that of gelatin chloride, while the curve for gelatin oxalate should be almost as high as that for gelatin chloride. Figure 38 shows that neither expectation is fulfilled.

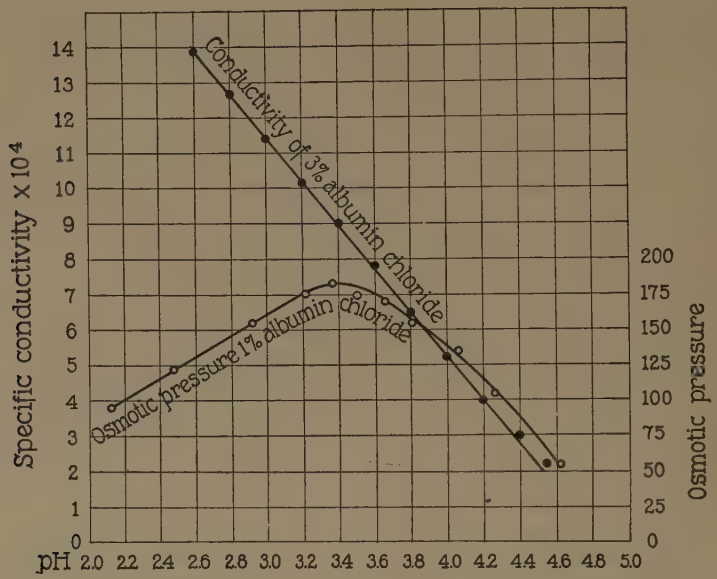
Specific conductivity x 10⁴
Osmotic pressure
pH
Conductivity of 3% albumin chloride
Osmotic pressure 1% albumin chloride
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
0 25 50 75 100 125 150 175 200
2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0

FIG. 39.—Comparison of conductivity curve and osmotic pressure curve for albumin chloride, showing the entirely different character of the two curves.

Figure 39 shows that the same disagreement exists between the conductivity curve and the osmotic pressure curve for solutions of the chloride of crystalline egg albumin. These curves, then, do not support the hydration hypothesis.

Pauli's hydration theory rests, as stated above, on an assumption made by Kohlrausch that the difference in the mobility of ions is due to molecules of water being dragged along with the migrating ion. Lorenz,[1] Born,[2] and others have come to the conclusion that while Kohlrausch's idea is probably correct for monatomic ions it cannot be correct for large polyatomic ions. This would

[1] LORENZ, R., *Z. Elektrochem.*, vol. 26, p. 424, 1920.
[2] BORN, M., *Z. Elektrochem.*, vol. 26, p. 401, 1920.

exclude the assumption of a high degree of hydration of protein ions.

The theory of adsorption is used to explain the precipitation of colloids by low concentrations of salts. The experiments described in the second, third, and fourth chapters of this book flatly contradict the assumption of such an adsorption when the concentration of salts is low.

The adsorption theory, the aggregate theory, and the hydration theory cannot explain the features of colloidal behavior enumerated at the beginning of this chapter.

As long as chemists continue to believe in the applicability of the adsorption formula to the behavior of proteins, no scientific theory of colloidal behavior will be possible. We intend to show in the second part of the book that such a theory can be given on the basis of the stoichiometrical proof that proteins form true salts with acids and alkalies, and that these salts lead to the formation of protein ions. Colloidal behavior is due to the fact that these protein ions cannot diffuse through many membranes which are permeable to the majority of crystalloidal ions, or that protein ions form solid gels in which cohesive forces prevent their diffusion, while such gels are permeable to crystalloidal ions. The theory of the equilibrium conditions resulting from this difference in the diffusibility of the two opposite ions of an electrolyte was developed by Donnan. These equilibrium conditions give rise to forces, such as P.D., osmotic pressure, etc., which are the only cause of colloidal behavior. It will be shown that Donnan's theory gives not only a qualitative but a quantitative and mathematical explanation of colloidal behavior.

CHAPTER VIII

MEMBRANE POTENTIALS[1]

We have seen that electrolytes influence the osmotic pressure, swelling, and viscosity of protein solutions in a similar way, so that we must think of the possibility that the cause of this influence is the same for all these properties.

When a solution of a protein salt, *e.g.*, 1 per cent gelatin chloride, is separated from distilled water by a collodion membrane, a potential difference exists across the membrane between the gelatin chloride solution and the outside solution with which it is in equilibrium. If this P.D. is measured with the aid of a Compton electrometer with saturated KCl calomel electrodes, it is found that the P.D. is influenced in the same way by electrolytes as the osmotic pressure, swelling, and viscosity (see Fig. 41 in this chapter). This in itself would only mean the addition of another property varying in the same characteristic way as osmotic pressure, or swelling, or viscosity of proteins under the influence of electrolytes, if it were not for the fact that we can correlate the variations of the new property with the Donnan equilibrium, and that we can calculate the P.D. with a fair degree of accuracy on the basis of this equilibrium. This then gives us a rational, quantitative theory of the influence of the pH, the valency of ions, and of the concentration of neutral salts on a colloidal property of proteins.

It is necessary to give a brief description of the method of measuring the P.D. Suppose that the protein in solution is gelatin chloride containing 1 gm. of originally isoelectric gelatin in 100 c.c. solution. Such solutions of gelatin chloride are put into collodion bags closed with rubber stoppers which are perforated with glass tubes serving as manometers, as described in the osmotic pressure experiments. These collodion bags filled

[1] This chapter is based on Loeb, J., *J. Gen. Physiol.*, vol. 3, pp. 557, 667, 1920–21; vol. 4, pp. 351, 463, 1921–22.

with the gelatin chloride solution are dipped into beakers containing 350 c.c. of aqueous HCl solution of originally the same pH as that of the gelatin chloride solution, but free from gelatin. The experiments last 20 hours or more at 24°C. to allow the establishment of osmotic equilibrium between the two solutions (which requires only about 6 hours under the conditions of the experiments). After 20 hours or more the P.D. between the gelatin solution (which we call the inside solution) and the aqueous

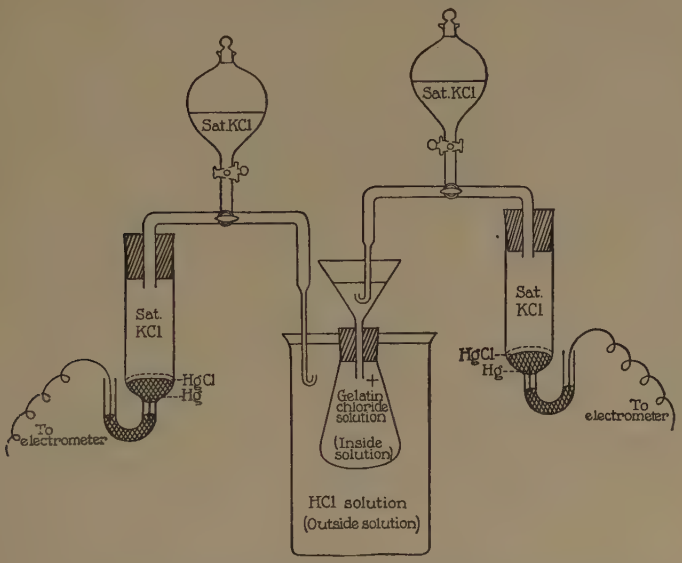

FIG. 40.—Method of measuring the P.D. between gelatin chloride solution in a collodion bag and the outside HCl solution in beaker.

solution (which we call the outside solution) is measured with the aid of a Compton electrometer, giving a deviation of about 2 mm. on the scale for 1 millivolt at a distance of about 2 m. The two electrodes leading to the electrometer are identical (Fig. 40). They are calomel electrodes filled with saturated KCl solution. One electrode dips through a capillary glass tube into the gelatin solution, the other also through a capillary glass tube into the outside solution. In order to allow the electrode to dip into the gelatin solution, the glass tube serving as a manometer is replaced by a funnel, as shown in the figure. In the figure the upper level

of the gelatin solution is in the funnel. This is not really necessary but it is convenient and is accomplished by allowing the collodion bag to press against the glass wall of the beaker con-

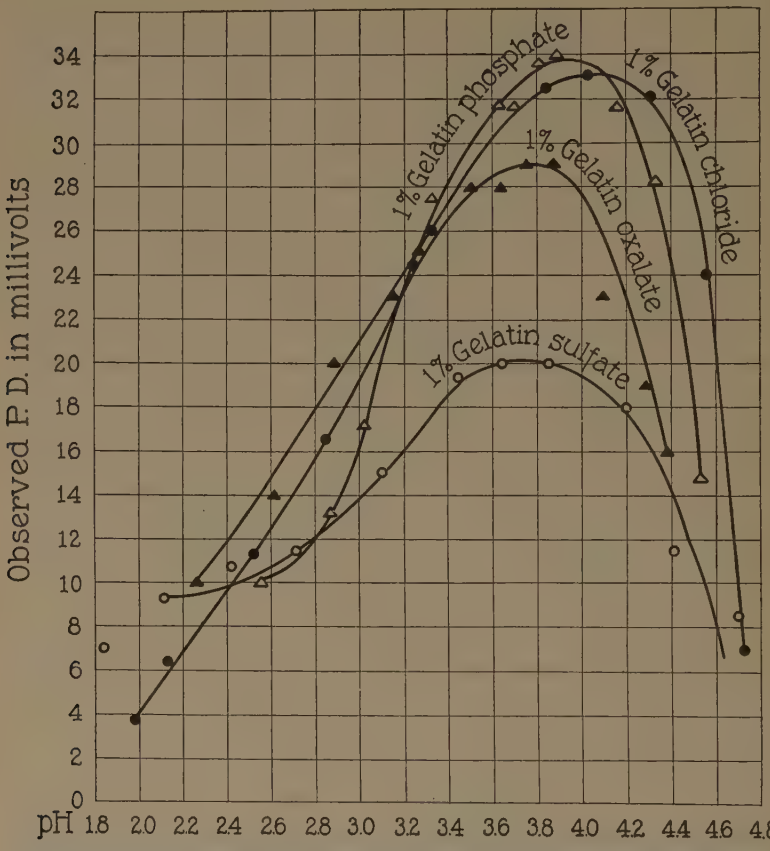

FIG. 41.—Influence of pH and valency of anion on P.D. of solutions of different gelatin-acid salts. The curves in Fig. 41 are similar to (but not identical with) those in Fig. 14.

taining the outside solution. As a minor but convenient accessory each electrode is connected with a reservoir of saturated KCl solution which makes it possible to let the KCl solution in the capillary flow out after each measurement, so that the electrode is always clean for each new measurement. What was

measured in this way was, therefore, the electromotive force of the following cell,

calomel electrode	saturated KCl	outside solution HCl −	collodion membrane	inside solution gelatin chloride +	saturated KCl	calomel electrode

It is found that in this cell the gelatin solution has a positive charge and the outside solution a negative charge and that the P.D. varies with the pH of the gelatin chloride solution, as indicated in Fig. 41. It is also found that the P.D. of gelatin phosphate solutions is practically identical with the P.D. of gelatin chloride solutions of the same pH and that both are considerably higher (about 50 per cent higher, as we shall see) than the P.D. of gelatin sulphate solutions. We shall also see that the addition of a neutral salt to the gelatin chloride solution depresses the P.D. In other words, electrolytes influence the P.D. between gelatin chloride solution and outside solution in a way similar to that in which they influence the osmotic pressure and the viscosity of the same solution. It becomes, therefore, of considerable importance to find out the origin of this P.D. We intend to show that the P.D. is due to the establishment of a Donnan equilibrium between the gelatin chloride solution and the outside aqueous solution (free from gelatin).

We have already given a brief outline of Donnan's membrane theory in the first chapter. In our experiment a collodion bag filled with a 1 per cent solution of gelatin chloride is dipped into a beaker containing a solution of HCl (without gelatin) of originally the same pH as that of the gelatin solution. In this case we have free HCl inside as well as outside, but in addition we have inside the collodion bag a gelatin chloride solution which ionizes into Cl and a positive gelatin ion. The gelatin ion is unable to diffuse through the collodion membrane but the H ions and Cl ions can diffuse freely through the membrane. Donnan has shown that in this case an equilibrium condition is established in which the product of the concentrations of the H and Cl ions in the outside solution equals the product of the concentrations of the H and Cl ions inside. This equilibrium is expressed by

the following equation, which was used by Procter and Wilson for the distribution of free HCl between a jelly of solid gelatin chloride and surrounding water, but which holds also for the case where the gelatin chloride is in solution and separated from the outside solution by a collodion membrane impermeable for gelatin ions,

$$x^2 = y(y + z) \qquad (1)$$

where x is the concentration of H and Cl ions in the outside solution, y the concentration of the H and Cl ions of the free acid inside the gelatin solution, and z the concentration of the Cl ions in combination with the gelatin. (For the sake of simplification complete electrolytic dissociation of HCl and gelatin chloride is assumed.) Since all the quantities in Equation (1) are positive, the concentration x of the hydrogen ions in the outside solution must be greater than the concentration y of the hydrogen ions in the inside solution; and the total concentration of the chlorine ions in the inside solution, $y + z$, must be greater than the concentration of the Cl ions in the outside solution, x. This difference in the distribution of the crystalloidal ions on the opposite sides of the membrane is caused by the fact that one type of ions (the protein ions) cannot diffuse through the membrane.

We now come to the most important point for the foundation of the theory of colloidal behavior. If it is true that the Donnan equilibrium is the cause of the P.D. between a gelatin chloride solution and the outside solution, the Donnan equilibrium is likely to be also the cause of the influence of the mysterious influence of electrolytes on the other properties of proteins, since the curves for P.D. are similar to the curves of osmotic pressure, viscosity, and swelling. In order to prove that the P.D. is due to the Donnan equilibrium, we must be able to show that the unequal distribution of the H and Cl ions on the opposite sides of the collodion membrane allows us to account quantitatively for the P.D. on the basis of Nernst's well known logarithmic formula for concentration cells.

We have seen in Chap. IV that we can determine the concentration of the Cl ions of the gelatin chloride solution by titration; and we can, of course, also determine the Cl of the outside watery solution by titration. Let x be the concentration of Cl

ions in the outside solution and $y + z$ the concentration of Cl in the gelatin solution (as found by titration) and let us assume that this difference of concentration determines the P.D. observed between the gelatin chloride solution and the outside solution, then we should expect that at 24°C. the observed P.D. = .059 $\log \frac{x}{y+z}$ volts.

We shall see later in this chapter that the observed P.D. in millivolts is actually equal to 59 $\log \frac{x}{y+z}$ millivolts, and this makes it very probable that the P.D. between a gelatin chloride solution and the outside watery solution across a collodion membrane is caused exclusively by the Donnan equilibrium.

We have a second check since it follows also that we must be able to calculate our observed P.D. on the basis of the difference in the concentration of hydrogen ions on the opposite sides of the membrane with the aid of Nernst's formula. Donnan's equilibrium equation

$$x^2 = y(y + z)$$

can be written in the form

$$\frac{y}{x} = \frac{x}{y+z}$$

where y is the concentration of the hydrogen ions inside the gelatin solution and x the concentration of the hydrogen ions in the outside solution. Hence, if the Donnan equilibrium is responsible for the observed P.D. between the gelatin chloride solution and the watery solution, it must also be possible to show that

$$\text{Observed P.D.} = 59 \log \frac{y}{x} \text{ millivolts}$$

We intend to show that this is actually the case. Instead of measuring the concentration of the hydrogen ions inside (*i.e.*, in the gelatin solution) and outside (*i.e.*, in the aqueous solution) by titration, we measure these concentrations with the hydrogen electrode. Since $\log y$ is the value pH inside and $\log x$ the value pH outside, the value 59 (pH inside minus pH outside) millivolts must (within the limits of accuracy of measurement) be equal to

the observed P.D. if the Donnan equilibrium is the exclusive cause for the P.D. between a gelatin chloride solution and the outside watery solution; neglecting the sign.[1]

Although the value pH inside minus pH outside is an observed value, *e.g.*, observed with the hydrogen electrode, we will call the value 59 (pH inside minus pH outside) the calculated P.D. to distinguish it from the P.D. observed with the indifferent electrodes.

The Influence of the Hydrogen Ion Concentration of Gelatin Solutions on the P.D.

Collodion bags of a volume of about 50 c.c. were filled with 1 per cent solutions of gelatin chloride of different pH. The bags were put into beakers containing each 350 c.c. of distilled water. To hasten the establishment of equilibrium between gelatin chloride solution and outside water some HCl was added to the latter —in fact the pH of the gelatin and the outside solutions was generally made equal at the beginning of the experiment. The collodion flasks containing the gelatin solution were closed with rubber stoppers which were perforated by glass tubes serving as manometers to allow the measurement of the osmotic pressure of the solution. After about 6 hours osmotic equilibrium was complete but we waited, as a rule, about 18 hours before measuring the P.D. across the membrane. Figure 41 shows that the pH influences the P.D. in a similar way as it influences the osmotic pressure, swelling, etc. Similar experiments were made with 1 per cent solutions of gelatin phosphate, gelatin oxalate, and gelatin sulphate, and the curves are also given in Fig. 41. To demonstrate the similarity between the curves for osmotic

[1] The sign of the observed P.D. was apparently, but not in reality, the reverse of the sign of the calculated P.D. In the "observed" P.D. the membrane (acting as a hydrogen electrode) was between the concentrated and dilute HCl, while in the "calculated" values the P.D. was obtained, from the potentiometric determinations of the pH. In this latter case two hydrogen electrodes were separated by a concentrated and a dilute solution. The "observed" P.D. was hence between two solutions of different concentrations while in the "calculated" values we measured the P.D. between two electrodes. In our tables the apparent (but not real) reversal of sign is corrected.

pressure and P.D., the osmotic pressures were observed in all the experiments used for measuring the influence of pH on the P.D., and Fig. 14 gives the osmotic pressures. A comparison of the two figures for P.D. (Fig. 41) and for osmotic pressure (Fig. 14) shows the following similarities: Both sets of curves rise from the isoelectric point with a lowering of the pH until they reach a maximum; this maximum is, however, not identical in the two cases. For the P.D. it varies between 3.6 and 4.0, while for osmotic pressure it lies near 3.5. With a further fall in pH both sets of curves show approximately the same steep drop.

The second point of similarity is the influence of valency. The curves for the P.D. (Fig. 41) are practically the same for gelatin chloride and gelatin phosphate, and are but slightly lower in the case of gelatin oxalate, while the curve for the P.D. is considerably lower in the case of gelatin sulphate. The same is true for the osmotic pressure curves (Fig. 14).

If these characteristic curves are exclusively determined by Donnan's membrane equilibrium it should be possible to show that the variation of the observed P.D. with pH is accompanied by a parallel variation of the value pH inside minus pH outside and that the agreement between these two sets of values is as perfect as the accuracy of the measurements permits. Tables XI, XII, and XIII show that this is true. The upper two horizontal rows give the pH inside and outside, the third horizontal row gives the difference, pH inside minus pH outside, and the fourth row gives the P.D. calculated in millivolts by multiplying the values pH inside minus pH outside by 59. The last horizontal row gives the observed P.D. in millivolts. The agreement between observed and calculated P.D. is sufficiently close to permit us to say that the characteristic curves representing the influence of the pH on the P.D. are a consequence of the Donnan equilibrium.

The Explanation of the P.D. Curve

Figure 41 shows that the P.D. of gelatin-acid salts is a minimum at the isoelectric point, that it rises rapidly with the increase in the hydrogen ion concentration until reaching a maximum at pH about 4.0 to 3.8, and then drops again with a further increase of

TABLE XI.—INFLUENCE OF THE HYDROGEN ION CONCENTRATION ON pH INSIDE MINUS pH OUTSIDE AND ON THE P.D. OF GELATIN CHLORIDE SOLUTIONS AT EQUILIBRIUM

	Cubic centimeters 0.1 N HCl added to 100 c.c. 1 per cent isoelectric gelatin											
	1	2	4	6	8	10	12.5	15	20	30	40	50
pH inside	4.56	4.31	4.03	3.85	3.33	3.25	2.85	2.52	2.13	1.99	1.79	1.57
pH outside	4.14	3.78	3.44	3.26	2.87	2.81	2.53	2.28	2.00	1.89	1.72	1.53
pH inside minus pH outside	0.42	0.53	0.59	0.59	0.46	0.44	0.32	0.24	0.13	0.10	0.07	0.04
P.D. calculated (millivolts)	+24.7	+31.0	+34.5	+34.5	+27.0	+25.8	+18.8	+14.0	+7.6	+5.9	+4.4	+2.3
P.D observed (millivolts)	+24.0	+32.0	+33.0	+32.5	+26.0	+24.5	+16.5	+11.2	+6.4	+4.8	+3.7	+2.1

TABLE XII.—INFLUENCE OF THE HYDROGEN ION CONCENTRATION ON pH INSIDE MINUS pH OUTSIDE AND ON P.D. OF GELATIN PHOSPHATE SOLUTIONS AT EQUILIBRIUM

	Cubic centimeters M/10 H_3PO_4 added to 100 c.c. 1 per cent isoelectric gelatin													
	0	1	2	4	6	7	8	10	12.5	15	20	30	40	50
pH inside	4.79	4.54	4.31	3.98	3.68	3.56	3.38	3.24	3.02	2.67	2.42	2.12	1.92	1.74
pH outside	4.70	4.10	3.77	3.40	3.14	3.04	2.90	2.80	2.66	2.39	2.22	1.98	1.83	1.67
pH inside minus pH outside	0.09	0.44	0.54	0.58	0.54	0.52	0.48	0.44	0.36	0.28	0.20	0.14	0.09	0.07
P.D. calculated (millivolts)	+5.3	+25.8	+31.7	+34.0	+31.7	+30.5	+28.0	+25.8	+21.2	+16.4	+11.7	+8.2	+5.3	+4.1
P.D. observed (millivolts)	+5.7	+27.0	+29.0	+30.0	+30.6	+29.6	+26.5	+24.4	+22.3	+17.7	+15.6	+11.4	+9.9	+7.3

TABLE XIII.—INFLUENCE OF THE HYDROGEN ION CONCENTRATION ON pH INSIDE MINUS pH OUTSIDE AND ON P.D. OF GELATIN SULPHATE SOLUTIONS AT EQUILIBRIUM

	Cubic centimeters 0.1 N H_2SO_4 added to 100 c.c. 1 per cent isoelectric gelatin													
	0	1	2	4	6	7	8	10	12.5	15	20	30	40	50
pH inside	4.76	4.52	4.34	3.98	3.73	3.49	3.41	3.12	2.78	2.47	2.16	2.06	1.84	1.57
pH outside	4.61	4.20	3.99	3.60	3.38	3.18	3.14	2.88	2.61	2.35	2.09	2.00	1.80	1.54
pH inside minus pH outside	0.15	0.32	0.35	0.38	0.35	0.31	0.27	0.24	0.17	0.12	0.07	0.06	0.04	0.03
P.D. calculated (millivolts)	+8.8	+18.8	+20.5	+22.2	+20.5	+18.1	+15.8	+14.0	+10.0	+7.0	+4.1	+3.5	+2.4	+1.8
P.D. observed (millivolts)	+6.3	+16.3	+18.4	+19.0	+19.0	+17.4	+15.8	+13.7	+10.5	+8.4	+7.4	+5.8	+4.7	+3.7

the hydrogen ion concentration. This is the characteristic pH effect already discussed in connection with viscosity, osmotic pressure, etc. Pauli explained this behavior of the viscosity curves on the basis of ionization and hydration. The hydration can have no connection with the P.D., but the ionization unquestionably has. Pauli assumes that the viscosity rises with increasing ionization of a protein salt and explains the maximum and drop by the repression of ionization of the protein salt by the anion of the acid added. This latter explanation is plainly inadequate in our case, since it would mean that the electrolytic dissociation of gelatin chloride is markedly diminished by a N/10,000 HCl solution. We can show that the Donnan theory accounts mathematically for the influence of pH on the P.D. of gelatin chloride solutions as expressed in Fig. 41.

Donnan had arrived at his equilibrium equation on the basis of thermodynamical considerations, but Proctor and Wilson have pointed out[1] that it can be derived more simply from the ordinary laws of ionization,

"since the nonionized portion of hydrochloric acid which, although small, must exist takes no direct part in the equilibrium and must be equal in both places."

Hence when a solution of gelatin chloride is separated by a collodion membrane from a solution of HCl (without gelatin), if x is the concentration of H and Cl ions in the outside solution and [HCl] the concentration of non-ionized HCl,

$$x^2 = \mathrm{K}\,[\mathrm{HCl}]$$

If y is the concentration of hydrogen ions inside the solution, y is the corresponding concentration of Cl ions; and if z is the concentration of Cl ions in combination with gelatin

$$y(y + z) = \mathrm{K}\,[\mathrm{HCl}]$$

hence

$$x^2 = y(y + z) \qquad (1)$$

$$x = \sqrt{y(y + z)}$$

[1] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, vol. 109, p. 309, 1916.

Substituting $\sqrt{y(y+z)}$ for x in the term $\frac{x}{y}$ we get

$$\frac{\sqrt{y(y+z)}}{y} = \sqrt{\frac{y+z}{y}} = \sqrt{1+\frac{z}{y}}$$

Hence at 18°C. the P.D. should be $= \frac{58}{2} \log\left(1+\frac{z}{y}\right)$ millivolts.

We will now show that from the term $\sqrt{1+\frac{z}{y}}$ the influence of pH on the P.D. as expressed in the curves of Fig. 41 can be derived.

When we add little HCl to isoelectric gelatin, we increase the amount of gelatin chloride formed and hence the value of z. Hence, the P.D. should increase since it depends on $\log\left(1+\frac{z}{y}\right)$. We also increase the value of y, but z and y will not increase at the same rate. z can be calculated from the equation $\frac{x}{y} = \frac{y+z}{x}$

$$z = \frac{x^2 - y^2}{y} = \frac{(x+y)(x-y)}{y}$$

When little acid is added to isoelectric gelatin the value of z rises more rapidly than the value of y, while when more acid is added the reverse happens. This is obvious from Table XIV comparing the variations of z and y upon the addition of increasing quantities of HCl to isoelectric gelatin.

TABLE XIV

Cubic centimeters 0.1 N acid in 100 c.c. 1 per cent originally isoelectric gelatin	pH of gelatin solution at equilibrium	Conc. y $\times 10^5$ N	Conc. z $\times 10^5$ N	$\frac{z}{y}$	$\log\left(1+\frac{z}{y}\right)$	P.D. calculated from $29 \log\left(1+\frac{z}{y}\right)$ millivolts	P.D. observed, millivolts
1.0	4.56	2.7	16.5	6.1	0.8513	24.7	24.0
2.0	4.31	4.9	51.4	10.5	1.0607	30.7	32.0
4.0	4.03	9.3	132.5	14.3	1.1847	34.4	33.0
6.0	3.85	14.1	200.0	14.2	1.1818	34.3	32.5
8.0	3.33	46.8	343.0	7.3	0.9191	26.1	26.0
10.0	3.25	56.2	372.0	6.6	0.8808	25.5	24.5
12.5	2.85	141.0	477.0	3.4	0.6435	18.7	16.5
15.0	2.52	302.0	608.0	2.0	0.4771	13.8	11.2
20.0	2.13	741.0	609.0	0.82	0.2601	7.5	6.4

The fifth vertical column of the table shows that at first the value $\frac{z}{y}$ increases with increasing addition of acid until pH = 4.03, and that with the addition of more acid the value $\frac{z}{y}$ diminishes again. A comparison of the last and second last vertical columns shows that the observed and calculated P.D. agree.

The Donnan equilibrium thus explains mathematically why the P.D. rises at first when HCl is added to isoelectric gelatin until the pH is 4.03, and why the P.D. drops when the pH falls below 3.8. No other colloidal theory is able to explain the curves in Fig. 41.

The Valency Effect

Figure 41 shows that the P.D. curves for gelatin chloride and phosphate are considerably higher than the curve for gelatin sulphate. The same valency effect was observed for the osmotic pressure curves, the viscosity curves, and the curves for swelling. It can be shown that the Donnan theory demands that for the same pH and the same concentration of originally isoelectric gelatin the P.D. of gelatin chloride and gelatin sulphate should stand in the exact ratio of 3:2, and it is one of the most convincing proofs of the correctness of the theory that the calculated values for the P.D. of these two gelatin salts agree with this postulate. By calculated values we mean the value 59 (pH inside minus pH outside).

The proof that the values for pH inside minus pH outside and, hence, the P.D. of the solutions of the two gelatin salts must show the ratio of 3:2 is as follows:

The equilibrium equation for gelatin chloride is of the second degree, namely,

$$\frac{x}{y} = \frac{y+z}{x}$$

and we have just seen that by proper substitution the

$$\text{P. D.} = \frac{58}{2} \log \left(1 + \frac{z}{y}\right) \text{millivolts.}$$

The equilibrium equation which is of the second degree when the anion is monovalent becomes of the third degree when the anion

is bivalent, *e.g.*, SO_4, in the case of gelatin sulphate. Let x be the concentration of hydrogen ions in the outside solution, y the hydrogen ion concentration in the inside solution; then $\frac{x}{2}$ is the concentration of the SO_4 ions in the outside, and $\frac{y}{2}$ the concentration of the SO_4 ions of the free H_2SO_4 in the inside (gelatin) solution. The concentration of SO_4 ions in combination with gelatin becomes $\frac{z}{2}$. Then the following two dissociation equations must hold:

$$x^2\frac{x}{2} = K\left[H_2SO_4\right] \text{ undissociated (outside)}$$

$$y^2\left(\frac{y}{2}+\frac{z}{2}\right) = K\left[H_2SO_4\right] \text{ undissociated (inside)}$$

Since the undissociated H_2SO_4 must be distributed equally on both sides of the membrane, $x^3 = y^2(y + z)$; $x = [y^2(y + z)]^{\frac{1}{3}}$. The value which interests us is $\frac{x}{y}$, *i.e.*, the ratio of the hydrogen ion concentrations.

Substituting $[y^2(y + z)]^{\frac{1}{3}}$ for x in $\frac{x}{y}$ we get

$$\frac{x}{y} = \sqrt[3]{\frac{y^2(y+z)}{y^3}} = \sqrt[3]{\frac{y+z}{y}}$$

The P.D. is, therefore, in the case of gelatin sulphate,

$$\text{P.D.} = \frac{58}{3}\log\left(1+\frac{z}{y}\right) \text{ millivolts}$$

while in the case of gelatin chloride it was

$$\text{P.D.} = \frac{58}{2}\log\left(1+\frac{z}{y}\right) \text{ millivolts}$$

Hence, the P.D. of gelatin sulphate solutions should be only two-thirds of the value of a gelatin chloride solution of the same pH and the same concentration of originally isoelectric gelatin.

This theoretical deduction is actually fulfilled, as the Tables XI, XII, and XIII show. Thus in Table XII we find that for

TABLE XV

Per cent 0.1 N HCl	100	99	98	96	90	80	60	40	20	10	0
Per cent 0.1 N H_2SO_4	0	1	2	4	10	20	40	60	80	90	100
Osmotic pressure, millimeters	436	420	425	420	415	365	315	275	235	218	202
pH inside	3.64	3.64	3.64	3.65	3.64	3.66	3.66	3.62	3.63	3.59	3.64
pH outside	3.15	3.15	3.13	3.13	3.15	3.17	3.23	3.23	3.29	3.27	3.33
pH inside minus pH outside	0.49	0.49	0.51	0.52	0.49	0.49	0.43	0.39	0.34	0.32	0.31
P.D. calculated, millivolts	+28.7	+28.7	+29.8	+30.4	+28.7	+28.7	+25.2	+22.8	+19.9	+18.7	+18.0
P.D. observed, millivolts	+28.5	+29.5	+29.5	+29.5	+27.5	+26.0	+24.5	+20.0	+19.0	+18.0	+16.0

gelatin phosphate of pH 3.98 the calculated P.D. was 34.0 and from Table XIII we notice that for gelatin sulphate of pH 3.98 the calculated P.D. was 22.2 millivolts. This is (within the limits of accuracy of the observations) the ratio 3:2, which the theory demands. For pH 4.31 the P.D. is 31 in the case of gelatin chloride and phosphate and for pH 4.34 it was 20.5 for gelatin sulphate, again the ratio of 3:2. The strict confirmation of the valency ratio came out clearly in the following experiment which was undertaken for another purpose—on account of its analogy with antagonistic salt action—but which shows that for the same pH the values of the calculated P.D. for gelatin chloride and sulphate are in the ratio of 3:2.

Solutions of 1 gm. of isoelectric gelatin were prepared in 100 c.c. of water containing 5 c.c. of a mixture of 0.1 N HCl and 0.1 N H_2SO_4 in various proportions. The solutions were put into collodion bags of about 50 c.c. volume and the latter were put into 350 c.c. of H_2O containing mixtures of N/1,000 HCl and N/1,000 H_2SO_4 in the same proportions as inside. The arrangement was that described for osmotic pressure measurements, and the osmotic pressure, P.D., and pH inside minus pH outside, were measured after 24 hours at 24°C. The upper rows of Table XV give

the ratio of cubic centimeters of 0.1 N HCl and 0.1 N H_2SO_4 in 100 c.c. of liquid. The rest of the table requires no further explanation. As in all gelatin-acid salt experiments, the agreement between observed and calculated P.D. is good.

Both P.D. and osmotic pressure are depressed the more the more HCl is replaced by H_2SO_4, but the depressing effect is greater in the case of osmotic pressure than in the case of P.D. The result of interest to us is the following: The value of pH inside minus pH outside was, for gelatin chloride of pH 3.64, = 0.49, and for gelatin sulphate of pH 3.64, = 0.31. The ratio of the two values is as nearly 3:2 as the accuracy of the measurements permits us to expect. The values for the observed P.D. agree with the values of the calculated P.D. within the limits of accuracy of the measurements.

This quantitative agreement between the observed P.D. and the P.D. calculated on the basis of the Donnan theory leaves little doubt that the observed P.D. is exclusively determined by the Donnan equilibrium.

Hydrogen Ion and Chlorine Ion Potentials

If we write the equation for the equilibrium condition between gelatin chloride solution and water in the form

$$\frac{y}{x} = \frac{x}{y+z}$$

where x is the concentration of H and Cl ions in the outside solution, y the concentration of the H and Cl ions of the free HCl inside the gelatin chloride solution, and z the concentration of the Cl ions in combination with the gelatin, $\frac{y}{x}$ is the ratio of hydrogen ion concentration inside to the hydrogen ion concentration outside; and $\frac{x}{y+z}$ the ratio of the concentration of the chlorine ion outside to the chlorine ion inside. Since,

$$\log \frac{y}{x} = \text{pH inside minus pH outside}$$

and

$$\log \frac{x}{y+z} = \text{pCl outside minus pCl inside}$$

it follows that

pH inside minus pH outside = pCl outside minus pCl inside (2)

If Donnan's membrane equilibrium is the cause of the influence of pH on the P.D. (and on the other physical properties) of protein solutions, we must be able to show that equation (2) is actually fulfilled.

This consequence of Donnan's theory was put to a test and some of the experiments described in the preceding part of this chapter were selected for this purpose. Inside the collodion bags were 1 per cent solutions of gelatin chloride of different pH; outside, water. After 18 hours equilibrium was established between inside and outside solutions and the pCl as well as the pH was ascertained. The pCl was determined in two different ways in the two experiments; in one experiment it was determined with the calomel electrode, in the other it was determined in the gelatin chloride solution by titration with NaOH according to the method described in the fourth chapter. Both methods of determining the pCl led to the result that the value pCl outside minus pCl inside was for the same solution at the point of equilibrium equal to the value pH inside minus pH outside (within the limits of accuracy of the experiments). The pCl outside was identical with the pH outside since the outside solution contained only free HCl. The values of pH were all determined potentiometrically (Table XVI).

TABLE XVI

Experiment 1. pCl determined by titration

pH of gelatin chloride solution at equilibrium	4.13	3.69	3.30	3.10	2.92	2.78	2.46	2.26	2.01	1.76
pH inside minus pH outside	0.56	0.58	0.50	0.49	0.44	0.44	0.33	0.23	0.15	0.10
pCl outside minus pCl inside	0.48	0.51	0.59	0.44	0.44	0.38	0.35	0.22	0.15	0.11

Experiment 2. pCl determined electrometrically

pH of gelatin chloride solution at equilibrium	4.04	3.92	3.78	3.61	3.46	3.16	2.73	2.36	2.04	1.73
pH inside minus pH outside	0.60	0.62	0.66	0.55	0.50	0.43	0.30	0.20	0.12	0.07
pCl outside minus pCl inside	0.55	0.60	0.57	0.50	0.53	0.38	0.32	0.17	0.12	0.07

Nernst's formula leads therefore to the same theoretical P.D. regardless of whether we calculate the P.D. on the basis of the difference pH inside minus pH outside or on the basis of the difference pCl outside minus pCl inside. It is also obvious that both assumptions lead to the same sign of charge of the gelatin chloride solution. If we assume that the P.D. is determined by differences in the hydrogen ion concentration, the outside solution is concentrated and the inside solution dilute; if the P.D. is determined by differences in the concentration of the Cl ions, the inside solution is concentrated and the outside solution dilute. Since the common ion is positive in the former and negative in the latter case, the gelatin solution becomes positive in both cases.

Outside Solution		Inside Solution	
$-$ H^+ concentrated	membrane	H^+ dilute	$+$
$-$ Cl^- dilute		Cl^- concentrated	$+$

The facts contained in this section of this chapter prove that the equation $x^2 = y(y + z)$ is the correct expression for the Donnan membrane equilibrium between acid-salts of proteins with monovalent anion and water, and that the Donnan equilibrium accounts for the P.D. observed. We wish to point out that we get the same result whether we determine pCl by titration or potentiometrically. The agreement with the theory is the same in both cases though the accuracy of the determination of pCl is less than that of pH.

The P.D. of Na Gelatinate

The Donnan theory demands that when a solution of Na gelatinate contained in a collodion bag is in equilibrium with a watery solution free from gelatin, free NaOH should be forced from the inside gelatin solution through the membrane into the outside watery solution free from gelatin. As a result the pH inside will now be less than pH outside, and the value pH inside minus pH outside will be negative for Na gelatinate while it was positive for gelatin chloride. If the Donnan equilibrium determines the P.D. (as it does) the sign of charge of Na gelatinate must be the reverse from what it was for gelatin chloride.

This is indeed the case and the turning point lies, as was expected, at the isoelectric point.

The experiments with Na gelatinate demand more rigid precautions than those with gelatin chloride. It is necessary to prevent the CO_2 of the air from diffusing into the alkaline solutions and therefore the outside solution was put into stoppered bottles connected with the outside air by glass tubes filled with soda lime. On account of the CO_2 error the pH measurements near the isoelectric point are unreliable and only when the pH is above 7.0 is it possible to get reliable results. The main facts demanded by the theory can, however, be demonstrated. The first fact is the proof that the sign of charge of the Na gelatinate solution is the reverse of that of a gelatin chloride solution.

Collodion bags of a volume of about 50 c.c. were filled with solutions of Na gelatinate containing 1 gm. of originally isoelectric gelatin and varying amounts of 0.1 N NaOH in 100 c.c. solution. The collodion bags were dipped into flasks containing 500 c.c. of aqueous solutions of NaOH of various concentrations and free from gelatin.

TABLE XVII.—1 PER CENT Na GELATINATE

Cubic centimeters 0.1 N NaOH added to 1 gm. gelatin in 100 c.c.	0	1	2	3	4	5	6	8	10	12.5	15	20
Concentrations of NaOH of outside solution	0	0	0	N/25,600	N/12,800	N/6,400	N/3,200	N/1,600	N/800	N/400	N/200	N/100
Osmotic pressure, millimeters	26	164	265	353	375	385	366	335	340	265	192	150
pH inside	5.02	5.40	5.76	6.64	7.15	9.02	9.68	10.16	10.45	...	11.30	11.58
pH outside	5.60	5.82	5.92	6.37	7.70	9.50	9.96	10.60	10.85	...	11.46	11.70
pH inside minus pH outside	− 0.58	− 0.42	− 0.16	+ 0.27	− 0.55	− 0.48	− 0.28	− 0.44	− 0.40	...	− 0.16	− 0.12
P.D. calculated, millivolts	−34.0	−24.5	− 9.4	+15.8	−32.0	−28.0	−16.5	−25.7	−23.4	...	− 9.4	− 7.0
P.D. observed, millivolts	− 3.5	−19.5	−18.0	−37.5	−37.5	−36.0	−30.0	−22.0	−19.5	...	−10.0	− 7.0

The flasks were sealed, communicating with the air only through tubes filled with soda lime, as stated. The collodion bags containing the gelatin were closed by a rubber stopper perforated by a glass tube which served as a manometer. The experiment lasted 6 hours at a temperature of 24°C. The results of the experiments are given in Table XVII. The upper horizontal row gives the number of cubic centimeters of 0.1 N NaOH originally in 100 c.c. of the gelatin solution; the second row gives the original concentration of NaOH in the outside aqueous solution free from gelatin; the third row gives the osmotic pressure in mm. H_2O after 6 hours. The next row gives the pH inside and the following row the pH outside after the experiment was finished (*i.e.*, after 20 hours), and the sixth row gives the difference pH inside minus pH outside. The reader will notice that this difference is always negative with one exception, which is obviously an error. The last two rows give the P.D. calculated from pH inside minus pH outside, and the P.D. observed.

It is obvious that there is no quantitative agreement between observed and calculated P.D. near the isoelectric point. As soon as the pH is above 7.0 the agreement between observed and calculated P.D. becomes better, so that we are entitled to say that the difference of potential between a Na gelatinate solution and an outside solution at or near equilibrium is due to the Donnan equilibrium which forces the expulsion of NaOH from the inside into the outside solution. As a consequence the pH inside becomes lower than the pH outside.

The Influence of Neutral Salts on the P.D. of Gelatin Chloride Solutions

It was shown in Chapter VI that the addition of neutral salts to solutions of protein salts depresses the osmotic pressure or viscosity of these solutions, and that the addition of neutral salts to a gel depresses the swelling of the latter (except when the solutions and gels are at the isoelectric point). It was of interest to find out whether or not the addition of a salt to a protein solution depresses also the P.D. across a collodion membrane, and whether this is also due to a depression of the value of pH

inside minus pH outside. It was possible to show that this is true.

Gelatin chloride solutions containing 1 gm. of originally isoelectric gelatin in 100 c.c. solution and having a pH of 3.5 were made up in different concentrations of $NaNO_3$ in water, the concentration of $NaNO_3$ varying from M/4,096 to M/32 $NaNO_3$, and all possessing a pH of 3.5. These mixtures were put into collodion bags and the bags were put into HCl solutions of pH 3.0 made up in different concentrations of $NaNO_3$, also of pH 3.0. These outside solutions contained no gelatin. The collodion bags were put into these outside solutions free from gelatin in such a way that the concentration of the $NaNO_3$ solution inside the collodion bag was always the same as outside.

When the P.D. across the collodion membrane was measured after 18 hours (after equilibrium was established) it was found that it was diminished upon the addition of neutral salt and the more the higher the concentration of the salt. This shows that the addition of neutral salt to a protein solution has a similar depressing effect on the P.D. as on the osmotic pressure, swelling, and viscosity of the protein solutions.

The next fact of interest was that the values of pH inside minus pH outside diminish in a parallel way with the diminution of the P.D. and that the values of 59 (pH inside minus pH outside) agree remarkably well with the observed P.D. (Table XVIII).

We have seen in Chapter VI that the addition of a salt with bivalent anion, *e.g.*, Na_2SO_4, to a gelatin chloride solution has a much greater depressing effect on the osmotic pressure, viscosity, etc., of the solution than the addition of a salt with monovalent anion, namely, $NaNO_3$. It can be shown that the addition of Na_2SO_4 also has a greater depressing effect on the P.D. of a gelatin chloride solution than has a $NaNO_3$ solution of the same molecular concentration (Table XIX).

We will consider as a third case the influence of $CaCl_2$ on the P.D. of a gelatin chloride solution. It has been shown that the depressing effect of $CaCl_2$ on the osmotic pressure of a gelatin chloride solution is about twice as great as that of an equimolecular concentration of NaCl. Table XX shows that the depressing influence of $CaCl_2$ on the P.D. is about twice as great as that of $NaNO_3$. The agreement between the observed P.D. and

TABLE XVIII.—DEPRESSING EFFECT OF NEUTRAL SALTS ON P.D. OF GELATIN CHLORIDE SOLUTIONS

	Concentration of $NaNO_3$								
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32
pH inside	3.58	3.56	3.51	3.46	3.41	3.36	3.32	3.29	3.25
pH outside	3.05	3.08	3.10	3.11	3.14	3.17	3.20	3.22	3.24
pH inside minus pH outside	0.53	0.48	0.41	0.35	0.27	0.19	0.12	0.07	0.01
P.D. calculated, millivolts	+31.2	+28.3	+24.0	+20.7	+16.0	+11.2	+7.0	+4.1	0
P.D. observed, millivolts	+31.0	+28.0	+24.0	+22.0	+16.0	+12.0	+7.0	+4.0	0

TABLE XIX.—DEPRESSING INFLUENCE OF Na_2SO_4 ON THE P.D. OF GELATIN CHLORIDE SOLUTIONS

	Concentration of Na_2SO_4											
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4
pH inside	3.54	3.41	3.35	3.32	3.29	3.30	3.33	3.38	3.41	3.41	3.37	3.29
pH outside	3.07	3.12	3.14	3.17	3.20	3.24	3.30	3.35	3.38	3.38	3.36	3.28
pH inside minus pH outside	0.47	0.29	0.21	0.15	0.09	0.06	0.03	0.03	0.03	0.03	0.01	0.01
P.D. observed, millivolts	+27.6	+17.0	+12.3	+8.8	+5.3	+3.5	+1.7	+1.7	+1.7	+1.7	+0.6	+0.6
P.D. calculated, millivolts	+26.5	+18.6	+12.5	+8.4	+4.7	+3.4	+1.5	0.0	0.0	0.0	0.0	0.0

TABLE XX.—DEPRESSING INFLUENCE OF $CaCl_2$ ON THE P.D. OF GELATIN CHLORIDE SOLUTIONS

	Concentration of $CaCl_2$										
	0	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
pH inside	3.55	3.45	3.41	3.36	3.30	3.28	3.26	3.25	3.25	3.25	3.22
pH outside	3.05	3.06	3.09	3.12	3.15	3.17	3.20	3.22	3.24	3.24	3.22
pH inside minus pH outside	0.50	0.39	0.32	0.24	0.15	0.11	0.06	0.03	0.01	0.01	0.0
P.D. calculated, millivolts	+29.5	+23.0	+18.9	+14.1	+8.8	+6.5	+3.5	+1.8	+0.6	+0.6	0.0
P.D. observed, millivolts	+28.6	+23.4	+19.2	+14.5	+9.1	+5.7	+3.1	+1.8	+1.1	+0.5	+0.5

the value 59 (pH inside minus pH outside), *i.e.*, the calculated P.D., is excellent.

It is of importance that the depressing effect of salts on the P.D. can be derived from the Donnan theory. To show this we must remember that the P.D. is expressed by the following term:

$$\text{P.D.} = \frac{58}{2} \log \left(1 + \frac{z}{y}\right) \text{millivolts}$$

When we add NaCl to a gelatin chloride solution we increase the concentration of the chlorine ions not in combination with gelatin, *i.e.*, y, while the concentration z of the Cl ions in combination with the gelatin remains the same, provided the pH remains the same (neglecting the diminution of ionization of gelatin chloride). Hence, the P.D. must become the smaller the greater y, and with steadily increasing y and constant z the value of $1 + \frac{z}{y}$ must approach 1; *i.e.*, the addition of enough salt must depress the P.D. to zero, which is actually the case.

This is also true when we add another salt, *e.g.*, $NaNO_3$, to a gelatin chloride solution. In this case we may assume that gelatin nitrate is formed.

The depressing effect of the addition of NaCl to gelatin chloride solution on the P.D. can be derived from the values of pH inside minus pH outside. The question arises, Why is it correct to neglect the influence of the Na ion? The writer did not give any reason for this but Dr. J. A. Wilson was kind enough to point out in a letter the mathematical proof justifying the writer's procedure in the following way:

"The true expression of the P.D. of a gelatin chloride solution the presence of NaCl is

$$\text{P.D.} = \frac{RT}{F} \log \frac{\left[H^+\right]\text{outside} + \left[Na^+\right]\text{outside}}{\left[H^+\right]\text{inside} + \left[Na^+\right]\text{inside}}$$

Let the system contain the positive ions A, B, C, etc., and the negative ions M, N, O, etc., whose concentration in the outside solution are, a, b, c, m, n, o, etc., and in the inside solution, a', b', etc. From the published work of Procter and Wilson it is evident that the product of concentration of any pair of oppositely charged ions is equal in both phases. The following equations are evident,

$$a \times m = a' \times m'$$
$$b \times m = b' \times m'$$
$$(a + b + c + \ldots)m = (a' + b' + c' + \ldots)m'$$
$$(a + b + c + \ldots)(m + n + o + \ldots) = (a' + b' + c' + \ldots)(m' + n' + o' + \ldots)$$

whence

$$\frac{a}{a'} = \frac{b}{b'} = \frac{c}{c'} = \frac{a + b + c + \ldots}{a' + b' + c' + \ldots}"$$

It is, therefore, immaterial which ion is singled out for the calculation of the P.D. on the basis of the Donnan effect. For the sake of the accuracy of measurement the hydrogen ion was selected.

It is perhaps worth while to point out that the agreement between calculated and observed P.D. is better in the experiments with salts than in the experiments without salts, especially near the isoelectric point. It seems almost as if the presence of too low a concentration of electrolyte increased the error of the measurements.

The Influence of the Sign of Charge

The fact that the P.D. of a protein-acid salt solution is a function of the term $\log (1 + \frac{z}{y})$, where z is the concentration of the anion in combination with the protein ions and y the concentration of the anion of the free acid, explains a phenomenon which is fundamental in colloidal behavior, namely, that whenever a salt depresses any physical property of a protein (or a colloidal solution in general) this action is due to that ion of the salt which has the opposite sign of charge to that of the protein ion. That this is true for the influence of salts on viscosity, osmotic pressure, and swelling has been discussed in Chap. VI, and we shall see that it is true also for the precipitation of certain protein solutions. In the latter case it is known as Hardy's rule of the precipitating action of salt. In all these cases the efficiency of the salt increases with the valency of the efficient ion of the salt. These rules are a consequence of the Donnan

equilibrium. The term derived from the equilibrium equation, $\log (1 + \frac{z}{y})$ makes the P.D. a function of z and y, *i.e.*, that ion which has the opposite sign of charge to the protein ion.

The Influence of the Concentration of Protein on the P.D.

While the addition of neutral salt depresses the P.D. of protein solutions across a membrane (as it depresses all the other properties) the addition of protein has the opposite effect, increasing the P.D. (as it increases also the other properties). This influence of the concentration of the protein follows mathematically from the equilibrium equation. Since P.D. $= \frac{58}{2} \log (1 + \frac{z}{y})$ millivolts, it is obvious that if y remains constant (*i.e.*, if no salt is present and the pH remains the same) while z increases as a consequence of the increase of the concentration of protein, the P.D. must rise with the concentration, and this was found to be the case.

Collodion bags, connected with glass manometers in the way described, containing 50 c.c. of different concentrations of originally isoelectric gelatin varying from 0.125 per cent to 2 per cent and containing enough H_3PO_4 to bring the gelatin solution to a pH of 3.5 were put into beakers containing 350 c.c. H_3PO_4 solution of pH 3.5. In order to prevent dilution of the protein solution through osmosis, the glass manometers were filled at the beginning of the experiment with the same gelatin phosphate solution as that contained in the collodion bag, to that height which the osmotic pressure measured in preceding experiments amounted to. After about 20 hours the pH in the inside and the outside solutions and the P.D. across the membrane were measured. Some of the experiments were made in duplicate (Table XXI).

It is obvious, first, that the P.D. increases with the concentration of gelatin, and second, that the increase of P.D. observed agrees quantitatively with the increase calculated on the assumption of the validity of Donnan's theory.

The P.D. of Solutions of Crystalline Egg Albumin

The experiments mentioned thus far had all been done on gelatin. It was of importance to determine whether or not

TABLE XXI

	Per cent of gelatin in solution											
	2	2	1½	1½	1	1	¾	¾	½	½	¼	⅛
pH inside	3.64	3.66	3.60	3.60	3.65	3.66	3.60	3.60	3.61	3.62	3.57	3.47
pH outside	3.02	3.02	3.02	3.01	3.12	3.11	3.14	3.12	3.21	3.19	3.25	3.29
pH inside minus pH outside	0.62	0.64	0.58	0.59	0.53	0.55	0.46	0.48	0.40	0.43	0.32	0.18
P.D. calculated	+36.6	+38.7	+34.2	+34.8	+31.3	+32.4	+27.2	+28.3	+23.6	+25.3	+18.8	+10.6
P.D. observed	+34.0	+36.5	+32.3	+34.0	+31.8	+32.3	+28.6	+28.6	+22.7	+22.7	+19.0	+13.0

TABLE XXII.—1 PER CENT ALBUMIN CHLORIDE

Cubic centimeters 0.1 N HCl added to 1 gm. albumin in 100 c.c.	0	1	2	3	4	5	6	7	8	10	15	20	30	40
Cubic centimeters 0.1 N HCl in 350 c.c. outside solution	0	0.1	0.3	0.5	1	1.5	2.1	2.8	4	7.1	16.4	32	60	80
Osmotic pressure in millimeters	155	100	52	114	178	205	214	219	218	180	138	100	81	74
pH inside	5.80	5.40	4.70	4.30	4.00	3.75	3.64	3.42	3.24	3.00	2.53	2.20	1.89	1.73
pH outside	6.14	5.64	4.67	4.06	3.65	3.38	3.22	3.07	2.91	2.71	2.37	2.10	1.82	1.70
pH inside minus pH outside	− 0.34	− 0.24	+ 0.03	+ 0.24	+ 0.35	+ 0.37	+ 0.42	+ 0.35	+ 0.33	+ 0.29	+ 0.16	+ 0.10	+0.07	+0.03
P.D. calculated, millivolts	−20.0	−14.0	+2.0	+14.0	+20.6	+22.4	+25.5	+21.0	+20.0	+17.5	+ 9.4	+ 6.0	+4.0	+2.0
P.D. observed, millivolts	−24.0	−16.0	+3.0	+11.5	+19.0	+19.5	+20.5	+19.5	+18.5	+16.0	+11.0	+10.0	+4.0	+3.5

these results could be confirmed with crystalline egg albumin. This was found to be the case, and the experiments on the membrane potentials of the solutions of the chloride of crystalline egg albumin showed a perfect quantitative agreement with the theory.

Collodion bags of about 50 c.c. volume were filled with a solution of 1 per cent crystalline egg albumin containing varying amounts of 0.1 N HCl, and the bags were put, as usual, into beakers containing 350 c.c. of HCl solutions of different concentration but free from albumin. The first two horizontal rows of Table XXII give the amount of 0.1 N HCl in each solution. The experiments were carried out at a temperature of 24°C., and after 22 hours the osmotic pressure, P.D., and pH of inside (albumin) solution and pH of the outside solution were measured. The albumin used was not isoelectric, but since it had been prepared after Sørensen's method it was probably partly ammonium albuminate, with a pH of near 6.0. The table shows that the calculated and observed P.D. agree beautifully (especially on the acid side of the isoelectric point); that the P.D. is a minimum near pH 4.70 of the albumin (*i.e.*, near the isoelectric point, which is at pH 4.8), and that the albumin is positively charged on the acid and negatively charged on the alkaline side of the isoelectric point. This is again in harmony with what we should expect on the basis of the Donnan equilibrium.

The next problem was to determine the influence of the addition of a neutral salt to a solution of the chloride of crystalline egg albumin. A 1 per cent solution of crystalline egg albumin containing 7 c.c. of 0.1 N HCl in 100 c.c. was made up in various concentrations of NaCl. The collodion bags containing these albumin chloride-NaCl mixtures were dipped into beakers containing 350 c.c. of the same concentration of NaCl as that of the albumin solution, and all made up in N/1,000 HCl. The experiment was carried out at 24°C. and the measurements were made after 22 hours.

Table XXIII gives the results, which show again a beautiful agreement between calculated and observed P.D.

We may, therefore, conclude that the P.D. of both gelatin solutions and solutions of crystalline egg albumin separated by a collodion membrane from a watery solution free from protein is

TABLE XXIII.—INFLUENCE OF SALT ON P.D. OF ALBUMIN CHLORIDE SOLUTION

	Concentration of NaCl									
	0	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
Osmotic pressure, millimeters	210	181	156	131	107	87	73	61	54	45
pH inside	3.35	3.32	3.32	3.27	3.25	3.20	3.19	3.22	3.21	3.22
pH outside	3.04	3.04	3.07	3.10	3.11	3.13	3.14	3.18	3.21	3.23
pH inside minus pH outside	0.31	0.28	0.25	0.17	0.14	0.07	0.05	0.04	0.00	−0.01
P.D. calculated, millivolts	+18.0	+16.2	+14.5	+10.0	+8.0	+4.1	+2.9	+2.3	0.0	−0.5
P.D. observed, millivolts	+18.5	+15.5	+13.5	+10.0	+7.5	+5.0	+3.0	+1.5	+1.0	+0.5

exclusively determined by the Donnan equilibrium, since otherwise the quantitative agreement between the observed P.D. and the values calculated from Donnan's equation would be impossible.

It may be stated, finally, that since P.D. $= 29 \log \left(1 + \frac{z}{y}\right)$ millivolts, it follows that the addition of a non-electrolyte to a gelatin chloride solution cannot influence the P.D. since the addition of a non-electrolyte cannot affect the value of $\frac{z}{y}$.

The expression for P.D. also explains why the P.D. is zero at the isoelectric point of a protein, since z becomes zero at that point. Moreover, it is obvious that the addition of a neutral salt to a solution of isoelectric gelatin cannot further depress the P.D. or cause a reversal in the sign of the charge.

Donnan's theory of membrane equilibrium therefore explains mathematically and quantitatively the P.D. observed at equilibrium between a protein solution and a watery solution free from protein, separated by a membrane. It does not happen very often that every postulate of a theory is fulfilled by the observation as it is in this case.

The bearing of this fact upon the theories of colloidal behavior is as follows: The P.D. is influenced by the hydrogen ion concentration, by the valency of the ion, by the addition of neutral salt in the same way as are the other properties of protein, such as osmotic pressure, viscosity, and swelling. Since the Donnan theory explains this influence of pH, valency, and salt effect on P.D. with mathematical accuracy, it seems at least highly probable that it may also explain the other colloidal properties of proteins in the same way, with this difference only, that the experimental error is much greater in the measurements of the other properties than in the measurements of P.D.

CHAPTER IX

THE ORIGIN OF THE ELECTRICAL CHARGES OF MICELLÆ, AND OF LIVING CELLS AND TISSUES

1. Stability of Suspensions, Electrical Charges of Micellæ, and Donnan Equilibrium

The stability of suspensions is, perhaps, the chief problem of a theory of colloidal behavior. Hardy[1] has shown that this problem is linked with the problem of the origin of the electrical charges of the particles in suspension, since such particles are forced by mutual electrostatic repulsion to remain in suspension. By his experiments on the migration of suspended particles of coagulated white of egg in an electrical field he proved that they have a positive charge in the presence of acid, a negative charge in the presence of alkali, and no charge at an intermediate point which he termed the isoelectric point of the particles. He was able to demonstrate that the stability of colloidal suspensions is a minimum at the isoelectric point.

He and others found, moreover, that low concentrations of neutral salts diminish the stability of colloidal suspensions in the presence of acids or alkalies and that the efficient ion of the salt has the opposite sign of charge to that of the colloidal particle, since the precipitating efficiency of a salt increases rapidly with the valency of that ion of the salt which has the opposite sign of charge to that of the colloidal particles. It seemed natural to infer that the precipitation of colloidal suspensions by low concentrations of a salt was caused by an annihilation of the charge of the colloidal particle. The problem of the stability of the colloidal suspension then developed into the problem of accounting for this peculiar behavior of the electrical charges of colloidal particles.

Hardy's original idea was that the H ions of the acid or OH

[1] Hardy, W. B., *Proc. Roy. Soc.*, vol. 66, p. 110, 1900.

ions of the alkali were adsorbed by the colloidal particle in preference to the other ions on account of their greater rapidity of migration;[1] and this idea was also accepted by Perrin[2] in his experiments on electrical endosmose, where it was necessary to account for the fact that certain membranes become positively charged in the presence of acid and negatively in the presence of alkali. Those who accept the adsorption hypothesis explain the fact that the electrical charges of the particles are apparently diminished or destroyed by the addition of a salt on the assumption of a preferential adsorption of one of the ions of the salt by the micellæ; yet such an assumption is incompatible with the purely stoichiometrical behavior of proteins. It is also difficult to account on the basis of the adsorption hypothesis for the fact that the addition of little acid increases while the addition of more acid depresses the electrical charge of micellæ.

A second possibility was pointed out by the writer in 1904,[3] namely, that Hardy's migration experiments might be explained in the case of proteins by the fact that proteins are amphoteric electrolytes which in the presence of alkali dissociate electrolytically by giving rise to a protein anion and in the presence of acid giving rise to a protein cation; while at the isoelectric point no protein ion would be formed. This idea could, however, not explain why the addition of a salt in low concentration should diminish the charge of aggregates of ions, *i.e.*, the micellæ, except by assuming that in this case the electrolytic dissociation of the protein salts should be repressed. The concentration of salts required for the precipitation of colloidal suspensions is, however, much too small to make such a suggestion acceptable. The idea is, however, correct if applied to the migration of isolated protein ions in the electrical field.

In 1916 J. A. Wilson[4] suggested that these electrical charges of micellæ were caused by the establishment of a Donnan equilibrium between the colloidal particle and the surrounding solution. There were, however, no measurements of membrane potentials

[1] Hardy, W. B., *J. physiol.*, vol. 29, p. xxvi, 1903.

[2] Perrin, J., *J. chim. physique*, vol. 2, p. 601, 1904; vol. 3, p. 50, 1905. Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918.

[3] Loeb, J., *Univ. Cal. Pub., Physiology*, vol. 1, p. 149, 1904.

[4] Wilson, J. A., *J. Am. Chem. Soc.*, vol. 38, p. 1982, 1916.

available at that time and this was probably the reason that his suggestion was not accepted.

We may consider a protein solution inside a collodion bag and surrounded by a watery solution as a model of a protein micella suspended in a watery solution. In that case it can be shown that the electrical charge of such a model varies in exactly the same way as the charges of colloidal particles in suspension, *e.g.*, coagulated egg albumin.

1. The electrical charge of the micella model (*i.e.*, gelatin solution in a collodion bag) is zero at the isoelectric point.

2. The charge of the model is positive on the acid side and negative on the alkaline side of the isoelectric point of gelatin and of crystalline egg albumin.

3. The charge of the model increases with the addition of little acid and diminishes with the addition of more acid to isoelectric particles.

4. The charge of the model is diminished by the addition of low concentrations of neutral salts, and the depressing action of the salt increases rapidly with the valency of that ion of the neutral salt which has the opposite sign of charge to that of the micella.

It has been shown in the preceding chapter that these facts can be explained not only qualitatively but quantitatively from the theory of Donnan's membrane equilibrium. This quantitative agreement leaves no doubt that the electrical charge of this micella model is caused exclusively by the Donnan equilibrium.

2. The Electrical Charge of Suspended Particles of Powdered Gelatin

We may ask whether it is justifiable to consider a gelatin solution enclosed in a collodion bag and surrounded by an aqueous solution as a model of a micella. This is theoretically correct since the colloidal behavior of both a true micella as well as the protein solution in a collodion bag is due to the same condition, namely, that the protein ion is prevented from diffusing into the outside aqueous solution while no such block exists for the diffusion of the crystalloidal ions. The block which prevents the diffusion of the protein ions in the model is the collodion membrane, while in the case of the micella (or a solid jelly) it is the

cohesion between the protein ions themselves. The formation of a micella depends upon these cohesive forces between the protein ions (or parts of these ions) becoming stronger than the attractive forces between the protein molecules and the molecules of water. The nondiffusibility of the protein ions of the micella must give rise to the establishment of a Donnan equilibrium between the micella and the surrounding liquid, and this equilibrium must give rise to the electrical charge of the micella. The only question is how far the agreement between Donnan's theory and the observed charge goes in the case of true micellæ. To test this we used suspensions of particles of powdered gelatin in water. If it can be shown, first, that these particles assume electrical charges when suspended in water, second, that these charges vary in the usual way with pH and the presence of salts, and third, that these charges can be derived from the Donnan equilibrium, the theory of these charges as well as the theory of the stability of colloidal suspensions can be put on an exact scientific basis.

The method of these experiments was as follows: Powdered particles of isoelectric gelatin were put into a solution of acid or alkali at a temperature of 20°C. and allowed to remain there for a number of hours to allow a complete or approximate equilibrium to be established between the inside of the micellæ and the outside solution.[1] The temperature must not be above 20°, since otherwise the granules of gelatin will dissolve too rapidly. After a number of hours the suspended particles were separated from the outside solution by filtration, the gelatin was melted and put into vessels with two bent tubes (see Fig. 42). After the gelatin had set to a gel (by cooling) the P.D. between the solid gel and the outside solution (filtrate) was determined with the electrometer. The P.D. was that of the following cell:

calomel electrode	saturated KCl	outside watery solution	solid gel	saturated KCl	calomel electrode

Everything else being symmetrical the P.D. measured was that between the suspended particles of gelatin (solid gel) and the outside solution with which the gelatin micellæ had been in complete or approximate equilibrium.

[1] Loeb, J., *J. Gen. Physiol.*, vol. 4, p. 351, 1921–22.

It can be shown that the P.D. between the micellæ and the surrounding solution is influenced in the same way by pH, valency, and salts as is the P.D. between a protein solution and a watery solution separated from each other by a collodion membrane. It was then necessary to ascertain whether this P.D. was due to Donnan equilibrium, and for this purpose the pH

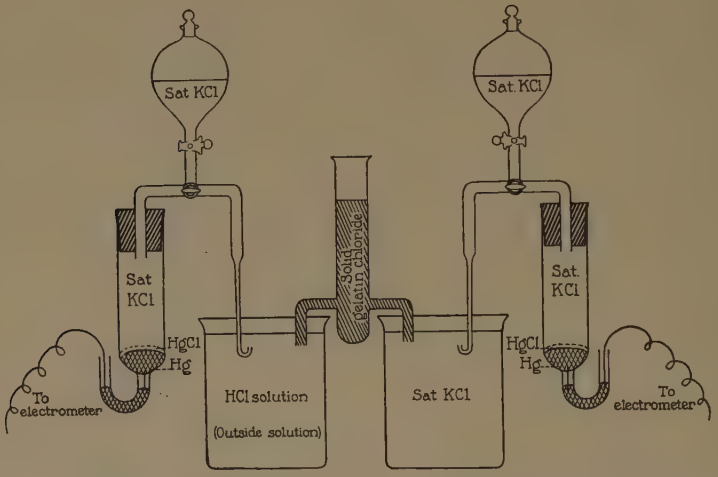

FIG. 42.—Method of measuring P.D. between gel and surrounding solution.

inside of the (melted) gelatin micellæ and the pH of the outside solution were measured with the hydrogen electrode and the potentiometer. It was found that the value 58 (pH inside the micellæ minus pH outside) agreed as closely with the P.D. observed with the Compton electrometer as the accuracy of the measurements permitted. This makes it highly probable that the electrical charge of the micellæ is determined by the Donnan equilibrium. We will again call the value 58 (pH inside micellæ minus pH outside) the calculated P.D. though it was in reality also observed. The accuracy of the P.D. measurements is not as great as in the case of the experiments of the preceding chapter, for reasons which we have not yet been able to ascertain.

3. The Influence of pH on the Charge of Suspended Particles of Powdered Gelatin

One-gram samples of powdered isoelectric gelatin going through mesh 30 but not through mesh 60 were put into 350 c.c. of water containing various quantities of HCl (see first horizontal row of Table XXIV), and left in these solutions for 24 hours at 20°C. The mixtures were occasionally stirred. After 24 hours the relative volume of the particles was measured and they were put on a filter to allow the acid to drain off. The gelatin was then melted by heating to 45°C. and poured into glass cylinders which at their lower end had two glass side tubes attached (Fig. 42). The mass was then allowed to solidify by cooling and the P.D. between gelatin and watery solution was ascertained. One of the two glass tubes dipped into a beaker containing the outside HCl solution (the filtrate) with which the gelatin had been in equilibrium, and the other dipped into a beaker containing a saturated solution of KCl. Each beaker was connected with a calomel electrode (filled with saturated KCl) leading to a Compton electrometer. The last row in Table XXIV gives the observed P.D. in millivolts.

The pH of the melted gelatin was determined potentiometrically. This is called pH inside in Table XXIV. The pH of the outside solutions (filtrate) was also determined.

While the agreement between the observed P.D. and the value of 59 (pH inside micellæ minus pH outside) is not as good as in the experiments with collodion bags, it is at least sufficient to leave no doubt that the charge is caused by the Donnan equilibrium in the way discussed in the preceding chapter.

4. The Influence of Acid and Alkali on the Sign of Charge of Micellæ

In Hardy's experiment with white of egg the particles were positively charged on the acid side of the isoelectric point and negatively charged on the alkaline side. It can be shown that this is also true for the charges of the suspended particles of powdered gelatin, and that this change of sign of charge of these particles by going from the acid to the alkaline side of the iso-

TABLE XXIV.—SUSPENSIONS OF POWDERED GELATIN

Cubic centimeters 0.1 N HCl in 100 c.c. H_2O.	0.5	1	2	4	6	8	10	12	15	20	30	40
Relative volume of gel	30	40	62	73	75	73	66	64	54	50	41	37
pH of melted gelatin (inside)	4.58	4.27	3.76	3.26	2.92	2.57	2.41	2.29	2.11	1.96	1.78	1.59
pH of supernatant liquid (outside)	3.89	3.45	3.04	2.65	2.44	2.27	2.16	2.07	1.95	1.82	1.65	1.49
pH inside minus pH outside	0.69	0.82	0.72	0.61	0.48	0.30	0.25	0.22	0.16	0.14	0.13	0.10
P.D. calculated, millivolts	+40.7	+48.4	+42.5	+36.0	+28.4	+17.7	+14.7	+13.0	+ 9.5	+ 8.3	+7.7	+5.9
P.D. observed, millivolts	+37.5	+39.0	+38.0	+29.5	+22.0	+17.7	+17.7	+18.2	+17.0	+10.7	+8.6	+5.4

TABLE XXV.—SUSPENSIONS OF POWDERED GELATIN

Cubic centimeters 0.1 N HCl or NaOH in 100 c.c. solution	HCl				NaOH						
	1.0	0.5	0.2	0.1	0	0.1	0.2	0.5	1.0	2.0	4.0
Relative volume of gelatin	28	20	18	16	17	18	28	37	40	47	48
pH of melted gelatin (inside)	4.44	4.56	4.79	4.85	4.89	4.98	5.06	5.50	6.74	9.54	10.15
pH of supernatant liquid (outside)	3.35	3.55	3.92	4.24	4.97	5.96	6.24	6.46	7.30	10.56	11.08
pH inside minus pH outside	+ 1.09	+ 1.01	+ 0.87	+ 0.61	0.08	− 0.98	− 1.18	− 0.96	− 0.56	− 0.02	− 0.93
P.D. calculated, millivolts	+63.0	+58.6	+51.0	+36.0	− 4.5	−57.0	−68.0	−56.0	−33.0	−59.0	−48.0
P.D. observed, millivolts	+56.0	+55.5	+36.5	+15.0	−17.5	−59.0	−61.0	−70.0	−66.0	−46.0	−36.0

electric point is accompanied by a change in the sign of the value (pH inside micellæ minus pH outside).

One gram of powdered gelatin of grain size between mesh 30 and 60, rendered isoelectric, was put into each of a series of closed flasks containing 350 c.c. of distilled water with varying quantities of 0.1 N HCl or NaOH per 100 c.c. (see Table XXV). The temperature was 20°C. After 4 hours the powdered gelatin was separated from its supernatant liquid by filtration, the gelatin was melted and the pH of the melted gelatin and of the outside solution (filtrate) were measured. The gelatin was then solidified and the P.D. between the solid gelatin and the filtrate (outside solution) determined, as described. The results of the experiments are given in Table XXV. The first row gives the number of cubic centimeters of 0.1 N HCl or NaOH contained originally in 100 c.c. outside solution. The next row gives the relative volume of the solid mass of gelatin, *i.e.*, the degree of swelling. The rest of the table needs no explanation. It is obvious that pH inside minus pH outside is positive as long as the pH of the gelatin is on the acid side of the isoelectric point, while it is negative when the gelatin is on the alkaline side of the isoelectric point. The turning point is approximately at the isoelectric point, but the measurements near the isoelectric point are obviously vitiated by experimental errors and possibly by some other factor, so that we cannot demonstrate more by the experiment than that suspended particles of solid metal gelatinate have the opposite sign of charge to that of the micellæ of gelatin chloride, and that this difference is accompanied by a reversal of the sign of the value of pH inside minus pH outside, which is positive in the case of gelatin chloride and negative in the case of Na gelatinate. It may also be pointed out that the minimum of swelling (volume) coincides with the minimum of P.D.

5. The Influence of Salts on the Charge of Suspended Particles of Gelatin

The most important fact which a theory of the electrical charge of colloidal micellæ is expected to explain is the annihilation of these charges by neutral salts. Those who believe in the adsorption theory assume that both ions of a neutral salt are adsorbed

by the colloidal particles, and that the salt ion with the opposite sign of charge to that of the colloidal particle diminishes the charge of the latter, while the salt ion with the same sign of charge as the colloidal particle increases the charge of the latter, and the more the higher the valency of the ion of the salt. The idea that such an adsorption occurs is definitely refuted by the experiments discussed in Chap. II (see Figs. 1 and 2). It might, however, be possible that the ion with the same sign of charge as the colloidal particle might increase the charge of the colloidal particle in some other way than through adsorption, and it was necessary to test this possibility, which has found acceptance on the part of some chemists. The writer's experiments on anomalous osmosis have shown that when a dilute solution of a salt is separated from pure water by a collodion membrane coated with gelatin, if the salt solution and the water are brought to the same pH by adding an acid, *e.g.*, HNO_3, the potential difference on the two opposite sides of the membrane increases with the valency of the cation of the salt used, *i.e.*, in the order

$$Ce > Ca > Na$$

This influence was found to be due to a diffusion potential.[1] Nevertheless it seemed necessary to determine whether or not these cations influenced the charge of suspended particles of gelatin chloride in the same way. If this were true, the depressing effect of $CeCl_3$ on the charge of the micellæ should be less than the depressing effect of $CaCl_2$, and the depressing effect of $CaCl_2$ should be less than the depressing effect of NaCl, provided the pH is on the acid side of the isoelectric point.

If, on the other hand, the Donnan effect alone determines the depressing effect of the salt on the charge of the suspended particles of gelatin, this depressing effect should be exclusively due to the anion of the salt on the acid side of the isoelectric point of the gelatin, while the cation of the salt should have no effect. This follows from the discussion in the preceding chapter according to which the P.D. between gelatin chloride solution and water is determined by the value of $\log\left(1 + \frac{z}{y}\right)$, where z and y are the anions. The cations do not enter into the term on

[1] Loeb, J., *J. Gen. Physiol.*, vol. 4, pp. 213, 463, 1921–22.

the acid side of the isoelectric point. We shall see that the measurements of the P.D. between micellæ and surrounding solution are sufficiently accurate to leave no doubt that the Donnan equilibrium alone determines the charge of the micellæ and that the cation of the salt does not increase the charge of the micellæ of gelatin chloride.

In order to get accurate measurements it was necessary to use micellæ of gelatin chloride of a pH sufficiently far from the isoelectric point to avoid the errors of the measurements which occur near that point. We weighed out doses of 1 gm. of powdered gelatin of a pH of near 7.0 and made them isoelectric by treatment with M/128 acetic acid and subsequent washing as described in Chap. II. In this process some gelatin was dissolved and lost (probably about 25 per cent). The isoelectric powdered gelatin was put into 200 c.c. of H_2O or a solution of different concentrations of a salt—NaCl, $CaCl_2$, $BaCl_2$, $CeCl_3$, or Na_2SO_4—and containing 16 c.c. of 0.1 N HCl. This brought the pH of the micellæ down to 2.8 or less, as Tables XXVI to XXX show. The powdered gelatin was left in these acid-salt solutions for two hours at 20°C., with frequent stirring. Then the supernatant liquid was separated from the powdered particles of gelatin by filtration and the P.D. between the micellæ and the surrounding liquid (filtrate) measured with the Compton electrometer using the electrodes described in Fig. 42. After this the value (pH inside − pH outside) was obtained with the aid of the hydrogen electrode at 24°C. and this value multiplied by 59 is called the calculated P.D. Tables XXVI to XXX give the results. The uppermost row gives the nature and concentration of the salt. The next row gives the relative volume of the gel of gelatin, and the depressing influence of the salt on the swelling; then follow the values for pH inside and outside measured with the hydrogen electrode and then the values pH inside minus pH outside. The last two columns give the calculated P.D., *i.e.*, the value 59 (pH inside minus pH outside), and the P.D. observed with the Compton electrometer and the indifferent electrodes described in Fig. 42.

The fact in common to all the experiments is the satisfactory agreement between the observed and calculated P.D. except that the calculated P.D. is on the average about 3 millivolts higher

TABLE XXVI.—INFLUENCE OF CONCENTRATION OF NaCl ON THE CHARGE OF MICELLÆ OF GELATIN CHLORIDE

	Concentration of NaCl											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
Relative volume of solid gelatin	49	49	47	46	45	44	40	40	38	29	25	25
pH inside	2.79	2.74	2.76	2.76	2.75	2.72	2.67	2.56	2.54	2.47	2.45	2.41
pH outside	2.28	2.28	2.28	2.28	2.28	2.28	2.28	2.29	2.31	2.33	2.33	2.34
pH inside minus pH outside	0.51	0.46	0.48	0.48	0.47	0.44	0.39	0.27	0.23	0.14	0.12	0.07
P.D. calculated, millivolts	+29.5	+26.6	+27.8	+27.8	+27.0	+25.5	+22.6	+15.6	+13.3	+8.0	+7.0	+4.0
P.D. observed, millivolts	+25.5	+23.0	+25.0	+25.0	+23.0	+21.5	+18.5	+14.0	+10.5	+7.0	+5.5	+2.5

TABLE XXVII.—INFLUENCE OF CONCENTRATION OF $CaCl_2$ ON THE CHARGE OF MICELLÆ OF GELATIN CHLORIDE

	Concentration of $CaCl_2$											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
Relative volume of solid gelatin	50	49	48	44	42	40	38	33	30	28	22	20
pH inside	2.80	2.80	2.75	2.77	2.72	2.71	2.65	2.59	2.53	2.49	2.47	2.43
pH outside	2.29	2.29	2.29	2.30	2.29	2.30	2.32	2.33	2.34	2.36	2.38	2.36
pH inside minus pH outside	0.51	0.51	0.46	0.47	0.43	0.41	0.33	0.26	0.19	0.13	0.09	0.07
P.D. calculated, millivolts	+29.5	+29.5	+26.7	+27.2	+25.0	+23.8	+19.1	+15.1	+11.0	+7.5	+5.2	+4.1
P.D. observed, millivolts	+25.5	+25.5	+23.0	+24.0	+21.0	+19.0	+15.5	+11.5	+ 7.5	+5.0	+3.0	+2.5

TABLE XXVIII.—INFLUENCE OF CONCENTRATION OF $BaCl_2$ ON THE CHARGE OF MICELLÆ OF GELATIN CHLORIDE

	Concentration of $BaCl_2$											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
Relative volume of solid gelatin	51	51	46	45	41	41	41	34	31	25	24	22
pH inside	2.80	2.77	2.73	2.73	2.73	2.67	2.65	2.57	2.55	2.51	2.47	2.44
pH outside	2.31	2.28	2.28	2.28	2.29	2.29	2.30	2.33	2.35	2.38	2.39	2.39
pH inside minus pH outside	0.49	0.49	0.45	0.45	0.44	0.38	0.35	0.24	0.20	0.13	0.08	0.05
P.D. calculated, millivolts	+28.5	+28.5	+26.0	+26.0	+25.5	+22.0	+20.0	+14.0	+11.5	+7.5	+4.5	+3.0
P.D. observed, millivolts	+26.0	+25.0	+24.0	+23.0	+22.0	+18.5	+15.0	+11.0	+ 7.5	+5.5	+2.5	+2.0

TABLE XXIX.—INFLUENCE OF CONCENTRATION OF $CeCl_3$ ON THE CHARGE OF MICELLÆ OF GELATIN CHLORIDE

	Concentration of $CeCl_3$										
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16
Relative volume of solid gelatin	51	50	48	46	43	42	42	35	32	25	24
pH inside	2.79	2.78	2.74	2.73	2.69	2.65	2.57	2.53	2.48	2.44	2.39
pH outside	2.31	2.29	2.29	2.29	2.28	2.32	2.33	2.35	2.35	2.38	2.36
pH inside minus pH outside	0.48	0.49	0.45	0.44	0.41	0.33	0.24	0.18	0.13	0.06	0.03
P.D. calculated, millivolts	+28.0	+28.5	+26.0	+25.5	+23.8	+19.0	+14.0	+10.5	+7.5	+3.5	+1.8
P.D. observed, millivolts	+26.0	+25.0	+22.0	+21.5	+19.0	+16.0	+11.5	+ 8.0	+5.0	+2.5	+2.5

TABLE XXX.—INFLUENCE OF CONCENTRATION OF Na_2SO_4 ON THE CHARGE OF MICELLÆ OF GELATIN CHLORIDE

	Concentration of Na_2SO_4											
	0	M/8,192	M/4,096	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
Relative volume of solid gelatin	52	51	49	47	44	40	38	34	28	24	23	22
pH inside	2.80	2.75	2.75	2.74	2.69	2.68	2.66	2.66	2.68	2.75	2.83	2.92
pH outside	2.33	2.29	2.30	2.32	2.32	2.36	2.41	2.45	2.56	2.67	2.79	2.89
pH inside minus pH outside	0.47	0.46	0.45	0.42	0.37	0.32	0.25	0.21	0.12	0.08	0.04	0.03
P.D. calculated, millivolts	+27.3	+26.7	+26.1	+24.3	+21.5	+18.5	+14.5	+12.2	+7.0	+4.6	+2.3	+1.7
P.D. observed, millivolts	+25.0	+22.0	+22.5	+21.0	+18.0	+16.0	+11.5	+8.5	+6.5	+4.0	+3.0	+1.5

than the observed P.D. and the cause for this difference is unknown at present. It is, however, a constant difference and has therefore no relation to the nature of the salt used. When we use as a standard for comparison of the relative depressing effect of a salt the concentration required to depress the observed P.D. to about 10 millivolts, we find that the following concentrations of the five salts are required for this purpose.

NaCl about M/64
$CaCl_2$ slightly above M/128
$BaCl_2$ slightly above M/128
$CeCl_3$ between M/256 and M/128
Na_2SO_4 about M/256

The main fact is that the depressing effect of the four salts NaCl, $CaCl_2$, $BaCl_2$, and $CeCl_3$ is determined by the chlorine ion concentration, and that the valency of the cation has no influence. This leaves no doubt that the charge of the micellæ is an unequivocal function of the Donnan equilibrium.

The depressing action of Na_2SO_4 is about four times as great as that of NaCl.

If the precipitating effect of a salt on the stability of colloidal suspensions is due exclusively to the depressing effect of the salt on the P.D. between micellæ and surrounding liquid, only that ion

should have an effect on the precipitation which has the opposite charge to that of the micellæ; and this precipitating effect should increase with the valency of the active ion. The Hardy-Schulze and Linder-Picton rule of precipitation is, therefore, only the consequence of the Donnan equilibrium.

It is necessary to correct in this place an error which occurs frequently in the colloidal literature, namely, the statement that in the precipitation of colloidal suspensions by neutral salts the colloidal particles are brought to the isoelectric point by the salt. What happens is that by the addition of neutral salts the P.D. between suspended particles and liquid is diminished, and if enough salt is added completely annihiliated. This is due to the fact that as a consequence of the addition of the salt the value y in the term $\log\left(1+\frac{z}{y}\right)$, upon which the P.D. depends, increases.

When, however, the gelatin granules are brought to the isoelectric point of gelatin, *i.e.*, to pH 4.7, through a change in the hydrogen ion concentration, the P.D. between particles and surrounding liquid becomes also zero, but for a different reason, namely, because the gelatin is now no longer ionized at this point. In this case the P.D. becomes zero because z in the term $\log\left(1+\frac{z}{y}\right)$ becomes zero.

If the theory of the Donnan equilibrium is applied to these phenomena it becomes therefore obvious that the P.D. between colloidal particles and surrounding liquid can become zero in two different ways: first, by making the value of z in the term $1+\frac{z}{y}$ equal to zero, and this is only possible by bringing the hydrogen ion concentration of the solution to that of the isoelectric point of the protein (which in the case of gelatin is at pH 4.7); and second, by making y in the term $1+\frac{z}{y}$ very large, *i.e.*, by increasing the concentration of the ions having the opposite sign of charge to that of the colloidal particles, and this can be done at any pH by adding a neutral salt.

It is therefore entirely wrong to say that the salt causes the precipitation of the suspended particles by bringing them to the isoelectric point or that the isoelectric point of a protein is shifted

by the addition of a salt. The isoelectric point of a protein is a constitutional property of the protein which need not be and probably, as a rule, is not affected by the addition of a neutral salt, since it is that hydrogen ion concentration at which a protein dissociates equally as an acid and as a base.

Electrical endosmose, anomalous osmosis, and kindred phenomena are due to the fact that there is a P.D. between the liquid and the walls of the membrane through which the liquid diffuses. It is often assumed that this P.D. is due to the adsorption of ions by the membrane whereby the charge of the adsorbed ion is transferred to the membrane. The writer tested this idea by experiments with membranes which had received a coating of a protein. He found that at the isoelectric point of the protein which forms the coating no electrical transport of water occurs either in electrical endosmose or in anomalous osmosis.[1] This agrees with the idea that the charge of the liquid inside the pores of the membrane is due to the Donnan equilibrium between membrane and liquid.

Experiments on anomalous osmosis were made to test the idea whether or not salts can transfer an electrical charge to solid particles of gelatin as acids or alkalies can. In order to test this idea these experiments were made with liquids of pH 4.7, *i.e.*, at the isoelectric point of gelatin. In this case the gelatin is not charged through acid or alkali and no electrical transport of water occurs at this point in either electrical endosmose or in anomalous osmosis. If now a salt, like $CaCl_2$ or Na_2SO_4, were able to transfer a charge to the gelatin, this should betray itself by an electrical transport of water through the gelatin membrane at pH 4.7 in experiments on anomalous osmosis. It was found that salts, like LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, Na_2SO_4, and others, at pH 4.7, leave the isoelectric gelatin uncharged, and that no electrical transport of liquid occurs at pH 4.7 in the presence of these salts. When, however, solutions of salts with trivalent cations, such as $LaCl_3$ or $CeCl_3$, or with tetravalent anion, like $Na_4Fe(CN)_6$, (all of pH 4.7) were used, the film of isoelectric gelatin assumed a charge; this charge was positive in the case of $CeCl_3$ or $LaCl_3$ and negative in the case of $Na_4Fe(CN)_6$. It was

[1] Loeb, J., *J. Gen. Physiol.*, vol. 2, p. 557, 1919–20; and in unpublished experiments.

apparently not due to a change in the pH since it occurred also when the salt solution was buffered by the addition of a mixture of Na acetate and acetic acid.[1] Perrin had noticed in his experiments on electrical endosmose[2] that salts with trivalent cations reversed the sign of charge of negatively charged membranes and that tetravalent anions reversed the sign of charge of positively charged membranes.

As a possible explanation the writer suggested a loose combination between isoelectric gelatin and the salts with trivalent cations or tetravalent anions, resulting in the formation of complex and positively charged gelatin-Ce or gelatin-La ions or negatively charged gelatin-$Fe(CN)_6$ ions. In other words, salts with trivalent (and tetravalent?) cations would react with isoelectric gelatin somewhat like acids, and salts with tetravalent anions would react somewhat like alkalies, the former causing the formation of positively charged complex protein ions, the latter causing the formation of negatively charged complex protein ions,—with this difference, that the protein-acid salts and metal proteinates are much more stable than the complex salts formed with trivalent cations and tetravalent anions. The result would in both cases be the ionization of the protein salt, resulting in a Donnan equilibrium and P.D. between solid protein and water.

It should be added that the experiments in Chap. II show that the Ce or $Fe(CN)_6$ ions can be washed away very easily, so that their compounds with gelatin differ in this respect from the compounds of gelatin with acid or alkali.

The tendency of proteins to form durable films when in contact with solid bodies probably explains the phenomenon that the addition of a little gelatin keeps coarser particles in suspension which without the gelatin would rapidly settle. If in this case the gelatin forms a solid film on the surface of the particle the latter will assume an electrical charge as long as the liquid has a pH different from 4.7; since as long as the pH is either less or more than 4.7 the P.D. between water and the gelatin-coated particles will keep the latter from settling. When, however, the pH is 4.7, this protective influence of the gelatin must disappear.

[1] Loeb, J., *J. Gen. Physiol.*, vol. 4, p. 463, 1921–22.

[2] Perrin, J., *J. chim. physique*, vol. 2, p. 601, 1904; vol. 3, p. 50, 1905. Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918.

It is very interesting that this film formation of gelatin on collodion membranes occurs regardless of the pH of the solution. It is, therefore, not necessary that the gelatin (or protein) be ionized to form a film on collodion membranes.

Aside from the electrical charges, the osmotic pressure of the solution seems also to have an effect on the stability of the colloidal solution. We shall see that the Donnan effect demands also that the osmotic pressure be influenced in a similar way by the pH, the valency, and the presence of salt as is the P.D. The depressing effect of the salt on the difference of osmotic pressure inside and outside the micellæ may possibly be of more importance in the precipitation of colloidal suspensions than the depressing effect of the salt on the electrical charge of the micellæ. This is indicated by the fact that the difference in the depressing effect of NaCl and Na_2SO_4 is greater for osmotic pressure than for P. D.

6. The Origin of the Electrical Charges of Living Cells and Tissues

In his first paper on the theory of membrane equilibria Donnan suggested that the membrane potentials postulated by his theory might contribute towards an explanation of the action of nerves and even of electrical fish. In 1911 the writer suggested to Dr. Beutner that he investigate the P.D. between such organs as apples, or leaves of the rubber plant, and water, instead of the P.D. of muscles or nerves, which had usually been used by physiologists for this purpose. In these experiments Dr. Beutner made the important observation that the P.D. between the surface of an apple or a leaf was a maximum when the bounding liquid was pure water, while the P.D. was depressed when a salt was added to the water, the depressing effect on the P.D. increasing with the concentration of the salt.[1] MacDonald[2] had observed a similar phenomenon, namely, the increase in P.D. between nerve and surrounding salt solution with increasing dilution. Donnan's theory was not known to us and we were not able to give an explanation of the depressing effect of salt on the P.D.

A search was made for those substances in the cortex of an apple or leaf which might be responsible for these peculiar con-

[1] Loeb, J. and Beutner, R., *Biochem.-Z.*, vol. 41, p. 1, 1912.

[2] MacDonald, J. S., *Proc. Roy. Soc.*, vol. 67, p. 310, 1900.

centration effects on the P.D. When the P.D. between solid gels of gelatin and of coagulated egg albumin and water was investigated, no potential differences were observed, to the great surprise and disappointment of the writer, who had hoped that the investigations of the P.D. might lead to an explanation of the antagonistic ion effects in which he was then interested. It is possible that the negative results with protein were due to the fact that the measurements were accidentally made near the isoelectric point. On the other hand, it was found that there existed a P.D. at the boundary of lipoids (lecithin dissolved in guaiacol) which was depressed by the addition of salts and the more the higher the concentration of the salt.[1]

This analogy between lipoids and living cells gave us the impression that the proteins had no share in the potential differences observed between living tissues or living cells and watery solutions. The experiments recorded in this chapter leave no doubt that this conclusion was wrong; any ion in a cell or on its surface which cannot diffuse into the surrounding watery solution (no matter whether the ion is a protein or a fatty acid or some complicated lipoid or a complicated carbohydrate or even a crystalloid) can or must give rise to a P.D. which is depressed when a diffusible salt is added to the surrounding watery solution.

The idea that lipoids are the substances responsible for the P.D. of tissues led Beutner to an extensive and most interesting investigation of the P.D. at the boundary of water-immiscible substances and water.[2] He found always a depressing effect of the addition of salt. Beutner tried to explain this on the basis of differences in the electrolytic dissociation in the watery and the water-immiscible (oily) phase. Such an explanation cannot be applied to the experiments with protein solutions and yet these latter solutions also show the depressing effect of the addition of salt on the P.D. in a most striking way. In this latter case the depressing effect of the salt on the P.D. is due to the Donnan equilibrium, and there is no reason why the theory of membrane equilibria should not apply to the P.D. between

[1] Loeb, J. and Beutner, R., *Biochem.-Z.*, vol. 51, p. 288, 1913; vol. 59, p. 195, 1914.

[2] Beutner, R., "Die Entstehung elektrischer Ströme in lebenden Geweben," Stuttgart, 1920.

oily and watery phases, since this theory only demands that one ion of the oily phase should be prevented from migrating into the watery phase. Any lipoid ion would fulfill this postulate of the theory. The peculiarities of electrolytic dissociation found by Beutner in non-aqueous solutions must, however, influence the Donnan equilibrium in a secondary way, since this equilibrium depends upon ionization.

CHAPTER X

OSMOTIC PRESSURE[1]

1. Theoretical Statements

The characteristic features of colloidal behavior appear also in the case of the osmotic pressure of solutions of protein salts. If the Donnan equilibrium is actually the cause of this behavior, as the experiments on membrane potentials suggest, it must be possible to derive these features of the osmotic pressure quantitatively and mathematically from Donnan's equilibrium formula. It is the purpose of this chapter to show that this is possible on the basis of van't Hoff's theory of osmotic pressure. The methods of measuring the osmotic pressure have been described in Chap. V. Collodion bags, of a volume of about 50 c.c., are filled with a protein solution, while the outside solution is 350 c.c. of water into which diffuses some of the free acid of the protein-acid salt solution or some of the free alkali of the metal proteinate solution. In order to hasten the establishment of equilibrium between inside and outside, the pH of the outside solution was usually at the beginning of the experiment brought to the same pH as that of the protein solution by adding the same acid or the same base as that of the protein solution. Equilibrium was established after about 6 hours but the measurements were usually taken after about 20 hours. The solutions were kept at a constant temperature of 24°C. throughout the experiment.

A gelatin chloride solution contains free hydrochloric acid, gelatin chloride (which dissociates electrolytically like any other salt in watery solution), and non-ionogenic protein molecules. A 1 per cent gelatin chloride solution of about pH 3.5 is in equilibrium with a HCl solution (free from protein) of a pH of about 3.0, the solutions being separated by a collodion membrane.

The terms for the calculation of the osmotic pressure of gelatin solutions are the same as those used by Procter (1914)

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 691, 1920–21.

and by Procter and Wilson (1916)[1] for the calculation of the swelling (see Chap. XI). Since, however, the application of the theory is simpler in the case of osmotic pressure than in the case of swelling, it may be well to discuss osmotic pressure experiments first.[2]

Let y be the concentration of the H and Cl ions of the free HCl inside a gelatin chloride solution (containing 1 gm. of originally isoelectric gelatin in 100 c.c.), z the concentration of the Cl ions held by the gelatin ions, and a the sum of the concentrations of the gelatin ions and non-ionized molecules of gelatin. For the sake of simplification we assume complete electrolytic dissociation of the gelatin chloride and of the HCl. In this case the osmotic pressure of the inside solution is determined by

$$2y + z + a$$

Since, however, the outside solution is at equilibrium not H_2O but HCl solution—in the example selected a HCl solution of about pH 3.0—the *observed* osmotic pressure is the difference between the osmotic pressure of the inside solution and the osmotic counterpressure of the outside solution.

Let x be the concentration of the H ions in the outside solution, then the osmotic counterpressure of the outside solution is determined by $2x$.

Hence the observed osmotic pressure of the gelatin chloride solution is determined by

$$2y + z + a - 2x$$

The osmotic pressure is observed experimentally, y can be calculated from the pH inside, and x from the pH outside.

z can be calculated from Donnan's equilibrium equation

$$x^2 = y(y + z) \qquad (1)$$

$$z = \frac{(x + y)(x - y)}{y}$$

where x, y, and z have the significance stated above. The z thus calculated differs, however, from the z obtained from the

[1] Procter, H. R., *J. Chem. Soc.*, vol. 105, p. 313, 1914. Procter, H. R. and Wilson, J. A., *J. Chem. Soc.*, vol. 109, p. 307, 1916.

[2] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 691, 1920–21.

titration values, and this is probably the cause of a slight discrepancy between observed and calculated osmotic pressures. For the present we calculate z from equation (1).

a is unknown, and we therefore can only calculate for the present the values of

$$2y + z - 2x$$

If we express the theoretical osmotic pressure of a grammolecular solution in terms of millimeter pressure of a column of H_2O we get (with correction for a temperature of 24°C.)

$$22.4 \times 760 \times 13.6 \times \frac{297}{273} = 2.5 \times 10^5 \text{ mm.}$$

In other words, a theoretical pressure of 2.5 mm. H_2O corresponds to a concentration of 10^{-5} N. In the following tables all concentrations are expressed in terms of 10^{-5} N and hence we only need to multiply the values for $2y + z - 2x$ given in our tables by 2.5 to obtain the calculated osmotic pressure of the gelatin solution in mm. H_2O (neglecting the osmotic pressure of the gelatin ions and molecules).

Equation (1) holds in the case of solutions of all gelatin-acid salts with monovalent anion; *i.e.*, gelatin chloride, acetate, phosphate, tartrate, citrate, etc. When, however, the anion of a gelatin-acid salt is divalent, as in the case of gelatin sulphate, the equilibrium equation becomes one of the third degree, as has been stated in Chap. VIII. If x is the hydrogen ion concentration of the outside solution, the concentration of the SO_4 ions in the outside solution becomes $\frac{x}{2}$. If y is the concentration of the H ions of the free sulphuric acid in the inside solution, $\frac{y}{2}$ is the concentration of the SO_4 ions of the free acid inside the gelatin sulphate solution. In the case of gelatin chloride z represented the concentration of chlorine ions in combination with the gelatin; hence $\frac{z}{2}$ will represent the concentration of SO_4 ions in combination with the same number of gelatin ions.

The equilibrium equation, therefore, assumes in the case of gelatin sulphate the following form:

$$x^2 \cdot \frac{x}{2} = y^2 \frac{(y+z)}{2} \qquad (2)$$

From equation (2) it follows that

$$z = \frac{x^3 - y^3}{y^2}$$

The osmotic pressure of the gelatin sulphate solution should therefore be calculated from the following values (omitting the share of the osmotic pressure due to the gelatin molecules and ions).

$$\frac{3}{2}y + \frac{z}{2} - \frac{3}{2}x$$

2. The Calculated Curves for the Influence of pH and Valency

Solutions containing 1 gm. of originally isoelectric gelatin in 100 c.c. and containing different quantities of acid were prepared. Collodion bags cast in the form of Erlenmeyer flasks of 50 c.c. volume were filled with the 1 per cent solutions of a gelatin-acid salt and put into beakers containing 350 c.c. of H_2O. In order to accelerate the establishment of the equilibrium between inside and outside solutions a certain amount of acid was added to the outside water (*e.g.*, HCl in the experiments with gelatin chloride, H_3PO_4, in the experiments with gelatin phosphate, etc.). Each Erlenmeyer flask was closed with a rubber stopper perforated by a glass tube serving as a manometer. All this was described in more detail in Chap. V.

In Fig. 43 are plotted the values of the osmotic pressures of 1 per cent solutions of gelatin chloride, gelatin phosphate, and gelatin sulphate, calculated on the basis of equations (1) and (2); and Tables XXXI, XXXII, and XXXIII give the data on the basis of which the calculations are made. The abscissæ in Fig. 43 are the pH in the inside solution at the point of equilibrium, the ordinates are the values for osmotic pressure calculated from the equations referred to. Figure 44 gives the actually observed osmotic pressures in the same experiments which furnished the data for the calculated curves in Fig. 43. The reader will notice that the three curves plotted in Fig. 43

show not only the same qualitative characteristics as the curves for the observed osmotic pressures in Fig. 44, but show them almost quantitatively; except that a correction for the value of osmotic pressure due to the gelatin particles themselves has to be added, a point which will be discussed later. What is of im-

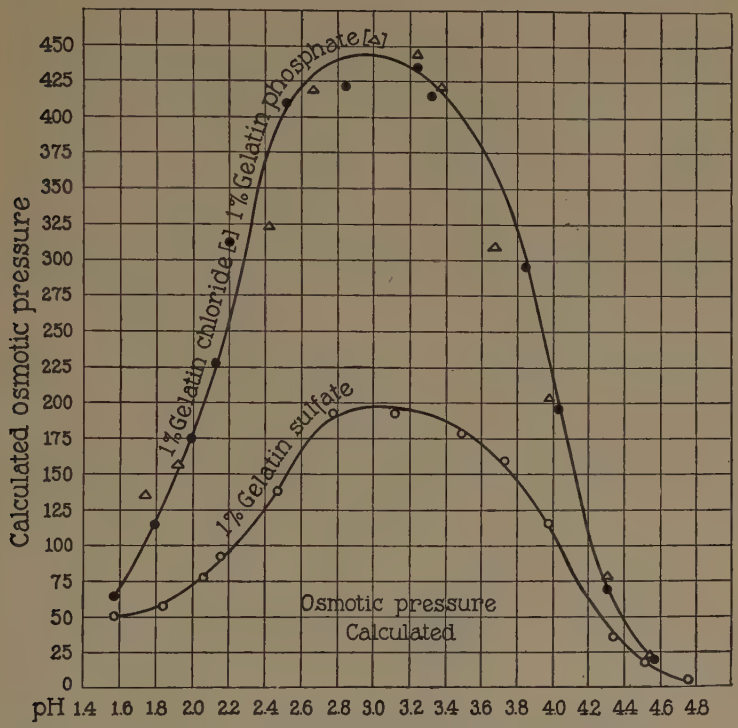

FIG. 43.—Calculated curves of osmotic pressure taken from the data of the experiments represented in Fig. 44. The calculation is made on the basis of the validity of Donnan's theory of membrane equilibrium. The calculations lead to curves resembling the curves in Fig. 44 in all essential points, in regard to valency effect of the anion, as well as in regard to influence of pH (see legend under Fig. 44).

portance here is the following: The curves for osmotic pressure calculated on the basis of the Donnan equation and plotted in Fig. 43 resemble the curves for the osmotic pressure observed in the same experiments represented in Fig. 44 in the following essential points.

(*a*) The curve for the calculated osmotic pressure of gelatin chloride is identical with the curve for the calculated osmotic pressure of gelatin phosphate, and the same is true for the two corresponding curves representing the observed osmotic pressures (Figs. 43 and 44).

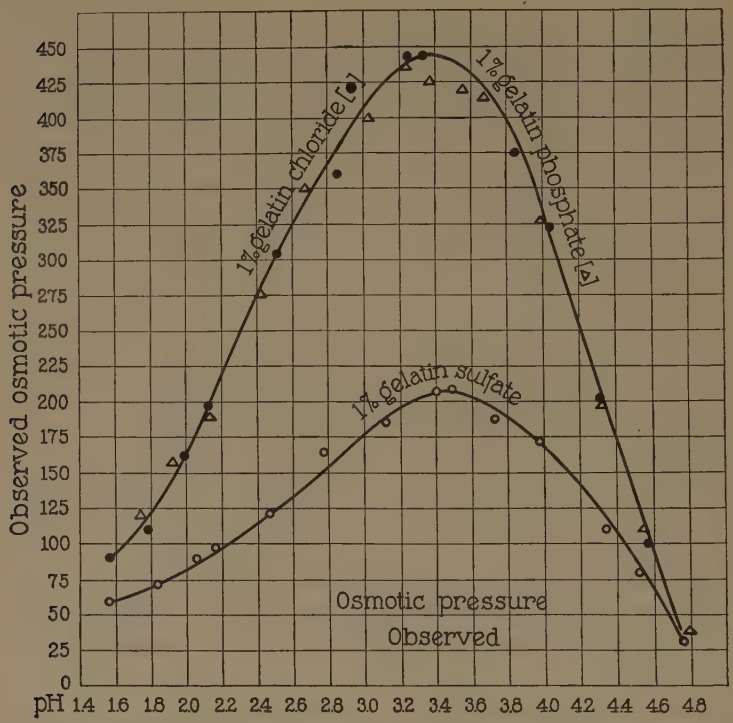

FIG. 44.—Observed curves representing the influence of pH and valency of anion on osmotic pressure of solutions in gelatin-acid salts containing 1 gm. of originally isoelectric gelatin in 100 c.c. solution. The curves for gelatin chloride and gelatin phosphate are identical since the anions, Cl and H_2PO_4, of these two gelatin salts are monovalent. The curve for gelatin sulphate is less than half as high as the curve for the two other salts because the anion of gelatin sulphate is bivalent. Both curves rise from the isoelectric point at 4.7 to a maximum at pH about 3.4 or 3.5, and then drop rapidly again.

(*b*) The curve for the calculated osmotic pressure of gelatin sulphate is a little less than half as high as the curves for the calculated osmotic pressures of gelatin chloride and gelatin phosphate; and the same is true for the curves representing the observed osmotic pressures of gelatin sulphate and gelatin chloride.

Table XXXI.—Gelatin Chloride

Observed and calculated osmotic pressures of gelatin chloride containing 1 gm. of originally isoelectric gelatin in 100 c.c. solution at equilibrium

pH inside	4.56	4.31	4.03	3.85	3.33	3.25	2.85	2.52	2.13	1.99	1.79	1.57
pH outside	4.14	3.78	3.44	3.26	2.87	2.81	2.53	2.28	2.00	1.89	1.72	1.53
$y = C_H$ inside $\times 10^5$	2.7	4.9	9.3	14.1	46.8	56.2	141.0	302.0	741.0	1,023.0	1,622.0	2,692.0
$x = C_H$ outside $\times 10^5$	7.2	16.6	36.3	54.9	135.0	155.0	295.0	524.0	1,000.0	1,288.0	1,905.0	2,951.0
$z = \frac{(x+y)(x-y)}{y}$	16.5	51.4	132.5	200.0	343.0	372.0	477.0	608.0	609.0	600.0	612.0	544.0
$2y + z - 2x$	7.5	28.0	78.5	118.4	166.6	174.4	169.0	164.0	91.0	70.0	46.0	26.0
Observed osmotic pressure	100.0	202.0	322.0	375.0	443.0	442.0	360.0	303.0	198.0	162.0	110.0	90.0
Calculated osmotic pressure, neglecting osmotic pressure of protein	19.0	70.0	196.0	296.0	416.0	436.0	422.0	410.0	227.0	175.0	115.0	65.0

Table XXXII.—1 Per Cent Gelatin Phosphate

Observed and calculated osmotic pressures at equilibrium

pH inside	4.79	4.54	4.31	3.98	3.68	3.56	3.38	3.24	3.02	2.67	2.42	2.12	1.92	1.74
pH outside	4.70	4.10	3.77	3.40	3.14	3.04	2.90	2.80	2.66	2.39	2.22	1.98	1.83	1.67
$y = C_H$ inside $\times 10^5$	1.6	2.9	4.9	10.5	20.9	27.5	41.7	57.5	95.5	213.8	380.2	758.6	1,202.0	1,820.0
$x = C_H$ outside $\times 10^5$	2.0	7.9	16.9	39.8	72.4	91.2	125.9	158.5	218.8	407.4	602.6	1,047.0	1,479.0	2,138.0
$z = \frac{(x+y)(x-y)}{y}$	0.9	18.6	53.3	140.0	228.0	231.0	338.0	380.0	405.0	556.0	575.0	686.0	617.0	690.0
$2y + z - 2x$	0.1	8.6	31.3	81.4	125.0	103.6	169.6	178.0	158.0	169.0	130.0	109.0	63.0	54.0
Observed osmotic pressure	34.0	111.0	199.0	328.0	416.0	420.0	426.0	436.0	401.0	350.0	275.0	190.0	158.0	121.0
Calculated osmotic pressure, neglecting osmotic pressure of protein	.....	22.0	77.0	203.0	310.0	258.0	423.0	445.0	395.0	420.0	324.0	273.0	157.0	135.0

TABLE XXXIII.—1 PER CENT GELATIN SULPHATE

Observed and calculated osmotic pressure at equilibrium

pH inside	4.76	4.52	4.34	3.98	3.73	3.49	3.41	3.12	2.78	2.47	2.16	2.06	1.84	1.57
pH outside	4.61	4.20	3.99	3.60	3.38	3.18	3.14	2.88	2.61	2.35	2.09	2.00	1.80	1.54
$y = C_H$ inside $\times 10^5$	1.7	3.0	4.6	10.4	18.6	32.3	38.9	75.9	166.0	339.0	692.0	871.0	1,445.0	2,692.0
$x = C_H$ outside $\times 10^5$	3.1	6.3	10.2	25.1	41.7	66.0	72.4	131.8	245.5	447.0	813.0	1,000.0	1,585.0	2,884.0
$z = \frac{x^3 - y^3}{y^2}$	8.3	24.7	45.8	136.0	191.5	243.0	212.0	322.0	390.0	435.0	433.0	449.0	466.0	620.0
$\frac{3}{2}y + \frac{z}{2} - \frac{3}{2}x$	2.0	7.35	14.5	46.0	64.0	71.0	55.8	77.0	77.0	55.0	37.9	31.0	23.0	20.0
Observed osmotic pressure	33.0	79.0	110.0	172.0	188.0	208.0	208.0	185.0	164.0	122.0	98.0	89.0	72.0	61.0
Calculated osmotic pressure, neglecting osmotic pressure of protein	5.0	18.5	36.0	115.0	160.0	178.0	.....	192.0	192.0	138.0	94.5	77.5	57.5	50.0

(*c*) All the curves in Figs. 43 and 44 rise from a minimum at pH 4.7, reach a maximum (which lies at pH 3.4 or 3.5 for the observed, and at 3.0 for the calculated curves), and then drop again as steeply as they rose on the other side. Moreover, the absolute values of observed and calculated osmotic pressures are almost equally high, a fact which will be discussed more fully a little further on.

It may be added that the curve for the calculated values of the osmotic pressure of gelatin oxalate solutions agrees also with the curve for the observed values of the osmotic pressure of solutions of the same gelatin salt, both being slightly lower than the curves for gelatin chloride.

In comparing the observed with the calculated values for osmotic pressure, the reader must keep in mind that the differences are exaggerated on account of the fact that the pressures are expressed in millimeters of a column of water instead of mercury. If we had expressed all the figures in terms of pressure of mercury, as is customary, the agreement would have appeared more complete.

We can, therefore, say that (with the exception of two minor discrepancies to be discussed further on) the Donnan equilibrium accounts not only qualitatively but almost quantitatively for (*a*) *the valency effect of the anion with which the gelatin is in combination;* (*b*) *for the effect of the pH.*

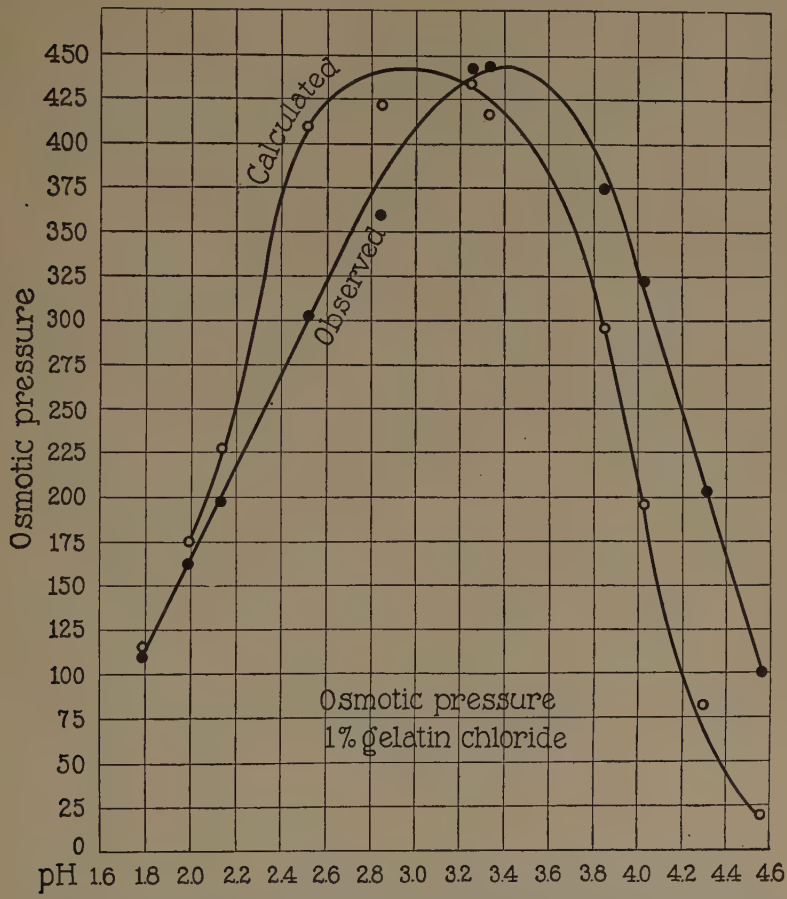

FIG. 45.—Showing agreement and minor discrepancies between the curves of observed and calculated osmotic pressures of 1 per cent gelatin chloride solutions.

A glance at the formulæ will show us that this influence of the pH on osmotic pressure is a mathematical consequence of

the theory. From the equilibrium equation, $x^2 = y(y + z)$, it follows that

$$x = \sqrt{y(y + z)}$$

If we substitute this value in the term for osmotic pressure $2y + z - 2x$, we get

$$2y + z - 2\sqrt{y(y + z)}$$

When z is zero (at the isoelectric point), the whole term becomes zero. At the isoelectric point we observe therefore the osmotic pressure of the protein solution free from the disturbing effects of the Donnan equilibrium. When we add acid to isoelectric gelatin, z increases and so does y, but, as we have shown in Table XIV of Chap. VIII, z increases at first more rapidly than y and later more slowly. Hence, the value of $2y + z - 2\sqrt{y(y + z)}$, *i.e.*, the osmotic pressure, increases at first the more acid we add to isoelectric gelatin until a maximum is reached. When y grows more rapidly than z, z becomes more and more negligible in comparison to y and the value of the term $2y + z - 2\sqrt{y(y + z)}$ diminishes again with increasing y, finally approaching zero again as a limit.

The curves representing the values for *calculated* osmotic pressures differ in one or two respects from the curves representing the values for the *observed* osmotic pressures (Fig. 45). As a rule, the calculated values are lower than the observed values (though this is only partly true for Fig. 45). This is to be expected since the calculated curves do not include that part of the osmotic pressure which is due to the protein particles and the calculated curves must therefore be too low, though this is (perhaps accidentally) not true for the descending part of the curve (for lower pH) in Fig. 45. The slight discrepancies between observed and calculated values may be due to an uncertainty in our calculations or to a simplification in our assumption which is not justified. We assume, *e.g.*, complete electrolytic dissociation of all compounds, which may not be entirely correct.

The discrepancies may also be due to an error in calculating z. We calculated z from $\frac{(x + y)(x - y)}{y}$ where x and y were the hydrogen ion concentrations determined electrometrically.

There is a second way of measuring z, namely, by determining the concentration of Cl inside a 1 per cent gelatin chloride solution by titration. The Cl inside is partly in combination with H (free HCl) and partly combined with gelatin. By titrating with NaOH to pH 7.0 and making the correction for isoelectric gelatin (as described in Chap. IV) we determine the value $z + y$. y is known from the pH and by deducting y, we get z. We made such determinations at the end of an osmotic experiment and calculated z also from $\frac{(x+y)(x-y)}{y}$ in the same experiment. Table XXXIV gives a comparison of the values of z obtained in identical solutions by the two different methods.

TABLE XXXIV.—CONCENTRATIONS OF $z \times 10^5$ N

pH of gelatin solution	4.51	4.26	3.96	3.61	3.53	3.32	3.23	2.86	2.32	2.16	1.93
z calculated from $\frac{(x+y)(x-y)}{y}$	30	90	166	223	252	316	387	493	570	687	687
z found by titration	17	84.5	170	275	291	342	401	532	548	838	885

There is no wide divergence between the two sets of values, yet enough to suggest that the calculated values of z may be chiefly responsible for the discrepancy between calculated and observed curves. The reader must remember that the value of z is multiplied by 2.5 in the calculations of the osmotic pressure (and, therefore, any error in the calculated osmotic pressure is multiplied in the same way).

3. The Influence of the Addition of Salts

It was first pointed out by R. S. Lillie that the addition of salt to a gelatin solution depresses its osmotic pressure. It should, however, be stated that this depressing effect does not occur at the isoelectric point. When we add different salts to a gelatin chloride solution of an initial pH 3.5 containing 1 gm. originally isoelectric gelatin in 100 c.c. solution, the depressing effect of the salt on osmotic pressure should according to the Donnan equa-

tion be due to the anion; and this is the case, as Fig. 46 shows. The gelatin chloride solutions were made up in different concentrations of the salts, NaCl, $NaNO_3$, $CaCl_2$, and Na_2SO_4. The pH of the mixtures was always 3.5. Collodion bags of a volume

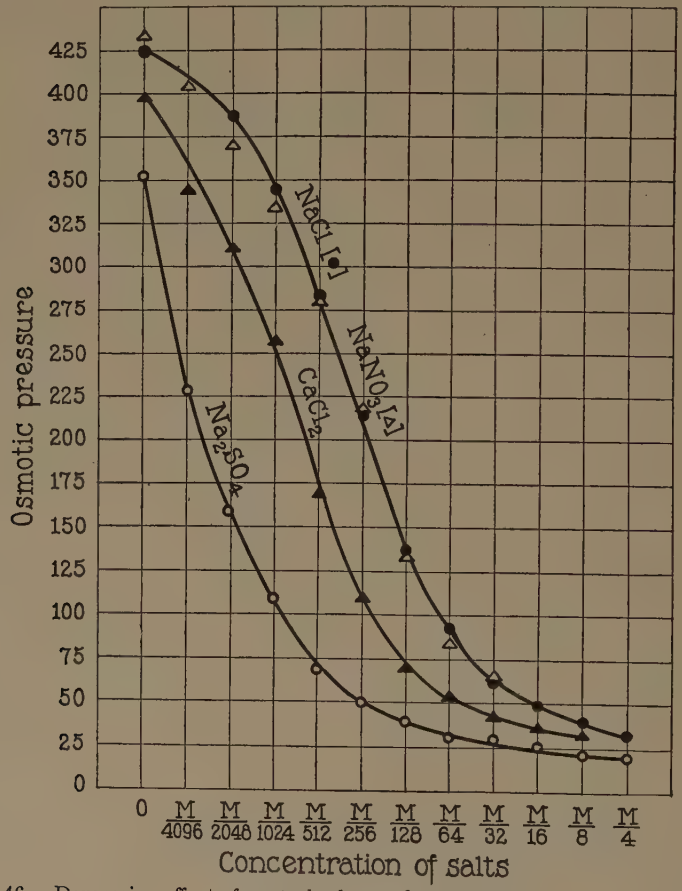

FIG. 46.—Depressing effect of neutral salts on the osmotic pressure of a 1 per cent solution of gelatin chloride of pH 3.5.

of about 50 c.c. were filled with the gelatin chloride-salt mixtures. These bags were dipped into beakers containing 350 c.c. of a solution of the same inorganic salt of the same concentration as that contained in the gelatin solutions, but these outside solutions contained no gelatin. The pH of the outside solutions

was made at the beginning 3.0 to accelerate the establishment of the equilibrium. The osmotic pressure was read after about 20 hours. The temperature was (as always in these cases) 24°C.

In Fig. 46 the abscissæ are the initial concentrations of the salt solutions while the ordinates are the osmotic pressures. The Donnan equilibrium caused a change of pH as well as of the distribution of the neutral salts on the opposite sides of the membrane. The change of pH in this experiment has already been discussed in Tables XVIII, XIX, and XX of Chapter VIII. Figure 46 shows that the depressing effect of NaCl and $NaNO_3$ is practically the same, that the depressing effect of an equimolecular concentration of $CaCl_2$ is not very far from twice as great as that of NaCl, but that the effect of Na_2SO_4—where the anion is bivalent—is about eight times as great as that of a NaCl solution of the same molecular concentration. This leaves no doubt that the depressing effect is due to the anion and that the cation is seemingly without any influence (it has certainly not any influence in the opposite direction from that of the anion). This depressing influence of the anion of a neutral salt on the osmotic pressure of protein-acid salts can be derived from the Donnan equilibrium equation.

Omitting that share of the osmotic pressure of the solution which is due to the protein molecules and ions, the share due to the Donnan equilibrium is expressed by the term

$$2y + z - 2\sqrt{y(y+z)} \qquad (1)$$

Suppose the gelatin be gelatin chloride and the salt added NaCl. Then z is the concentration of Cl in combination with gelatin, while y is the sum of the concentration of the Cl ions combined with the H ions of the free HCl present in the gelatin solution and the Cl ions of the NaCl contained in the gelatin solution at equilibrium. We can ascertain the total concentration of Cl ions inside the gelatin solution, *i.e.*, the value of $y + z$ in term (1) by titration. This term $2y + z - 2\sqrt{y(y+z)}$ will become the smaller, the more closely

$$\frac{2y+z}{2\sqrt{y(y+z)}}$$

approaches the value 1.

It is obvious that if z is small and constant, while y increases more and more (through the addition of NaCl), z becomes a negligible quantity and the term

$$\frac{2y+z}{2\sqrt{y(y+z)}} \qquad \text{approaches} \qquad \frac{2y}{2\sqrt{y^2}} = 1$$

We can measure the term $\sqrt{y(y+z)}$ directly by titrating the outside solution for Cl. We cannot determine $2y + z$ directly but we can determine $y + z$ by titrating the inside solution for Cl. If both titrations are made after equilibrium is established we get the value of

$$\frac{y+z}{\sqrt{y(y+z)}}$$

and the variations of this value with increasing concentration of NaCl are contained in Table XXXV. It is seen that this value is almost 1 when the NaCl solution is M/32.

Now the value of $\frac{y+z}{\sqrt{y(y+z)}}$ does not differ much from the value $\frac{2y+z}{2\sqrt{y(y+z)}}$ as long as y is large in comparison with z, and we can say that with z small and constant and y large and increasing rapidly, the two values

$$\frac{y+z}{\sqrt{y(y+z)}} \qquad \text{and} \qquad \frac{2y+z}{2\sqrt{y(y+z)}}$$

approach the value 1 almost (but not quite) at an equal rate. Hence, it follows from Table XXXV that if the concentration of NaCl becomes M/32 the value $2y + z - 2\sqrt{y(y+z)}$ must be nearly zero. In this case the osmotic pressure of the 1 per cent gelatin chloride solution must be almost but not quite down to that of the pure gelatin solution as it is at the isoelectric point. The actual observations plotted in Fig. 46 show that for M/32 NaCl or M/32 $NaNO_3$ a 1 per cent solution of gelatin chloride of pH about 3.5 has an osmotic pressure not far from that of isoelectric gelatin. If the values of $\frac{y+z}{\sqrt{y(y+z)}}$ are plotted as

ordinates over the values of the concentration of NaCl it is noticed that the two curves are approximately parallel (Fig. 47).

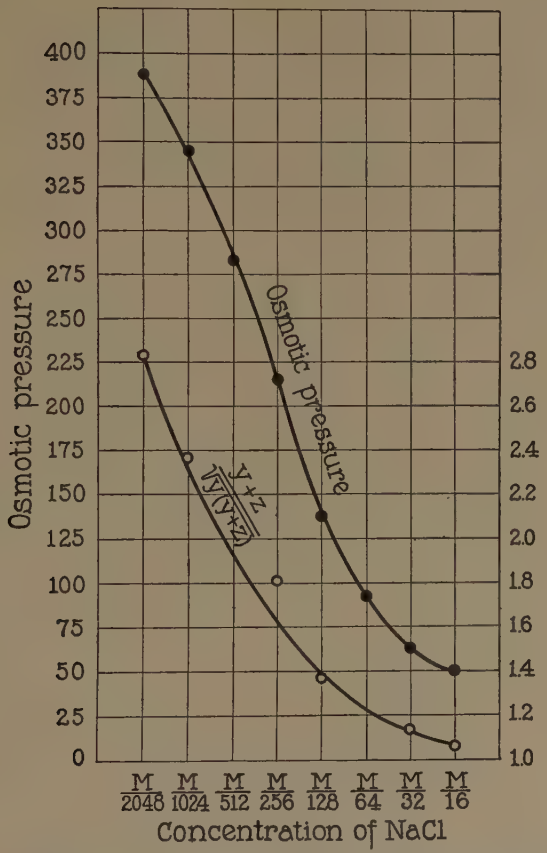

FIG. 47.—Parallelism between depressing action of NaCl in the osmotic pressure of a gelatin chloride solution and the curve representing the value $\frac{y+z}{\sqrt{y(y+z)}}$.

This shows that the Donnan equation actually accounts for the depressing effect of neutral salts on the osmotic pressure of a gelatin chloride solution.

TABLE XXXV.—INFLUENCE OF NaCl ON OSMOTIC PRESSURE OF 1 PER CENT GELATIN CHLORIDE SOLUTION.

Concentration of NaCl	Inside $y + z$	Outside $\sqrt{y(y+z)}$	$\frac{y+z}{\sqrt{y(y+z)}}$
M/2,048	566	200	2.83
M/1,024	633	267	2.37
M/512	(800	300	2.66)
M/256	966	534	1.81
M/128	1,370	1,000	1.37
M/32	3,800	3,340	1.14
M/16	6,930	6,540	1.06

4. THE INFLUENCE OF THE CONCENTRATION OF A PROTEIN SOLUTION UPON THE OSMOTIC PRESSURE

An increase in the concentration of a protein solution at the same pH and in the absence of neutral salts should have a double effect on the osmotic pressure. It should, first, raise the osmotic pressure of the solution on account of the increase in the number of protein particles in the solution; and it should, second, lead to a further increase in osmotic pressure due to an increase in the value of $2y + z - 2x$ or $2y + z - 2\sqrt{y(y+z)}$, for it is obvious that as long as y is constant, *i.e.*, at constant pH of the gelatin solution, the value of the term $2y + z - 2\sqrt{y(y+z)}$ will increase with increasing z. The two effects can be separated by subtracting the value of the term $2y + z - 2x$ from the observed osmotic pressure. The difference between the two values should (within the limits of the accuracy of the experiments) increase with the concentration of the protein. Both expectations are fulfilled.

Different concentrations of gelatin phosphate from 2 per cent to 0.5 per cent were prepared, all having a pH of 3.5. The gelatin phosphate solutions were put into encollodion flasks of 50 c.c. volume, each connected with a glass tube serving as a manometer as described, and these flasks were put into beakers containing 350 c.c. of H_2O, the pH of which was brought at the beginning of the experiment to 3.5 through the addition of H_3PO_4. When the bags containing gelatin phosphate solutions are put into water

the latter diffuses rapidly into the gelatin solution thereby lowering the concentration of the gelatin solution. To avoid this error so much gelatin phosphate solution was poured into each bag and glass tube that at the beginning of the experiment the liquid reached already to about that level which from preceding experiments we knew the gelatin solution would reach in the manometer at the point of osmotic equilibrium. All experiments were made in duplicate. In addition to the osmotic pressure we measured the pH inside and outside after equilibrium was reached. From these latter data the osmotic pressure due to the H and H_2PO_4 ions could be calculated, being equal to

$$(2y + z - 2x) \times 2.5 \text{ mm. } H_2O$$

By deducting this value from the observed osmotic pressure in each case it was hoped to obtain a rational value for the share of the protein particles in the observed osmotic pressure. Table XXXVI gives the results.

The reader's attention is called to the last two rows of figures (Table XXXVI) giving the difference between the observed and the calculated osmotic pressures, since if this difference actually represents the osmotic pressure due to the gelatin particles, the figures should be in direct proportion to the concentration of the gelatin. The experiments were all made in duplicate to give some idea of the magnitude of error, and it is obvious that the error may be considerable, 25 per cent or more, because the errors in the observed and the calculated values are additive. Thus the "difference" is for 0.75 per cent solution in one case 92, in the other 61, a variation of 50 per cent! If we take this into consideration we may conclude that the differences between the observed and the calculated osmotic pressures are compatible with the idea that the difference is the value for the osmotic pressure due to the gelatin particles in solution.

This would lead us to the conclusion that the osmotic pressure due to the gelatin particles in a 1 per cent solution (of originally isoelectric gelatin) of gelatin phosphate of pH 3.60 is about 100 mm. H_2O. Since the osmotic pressure of one grammolecule is about 250,000 mm. H_2O and since 1 liter of a 1 per cent solution of gelatin contains 10 gm. of gelatin, the molecular weight of gelatin should be expected to be in the neighborhood of 25,000.

TABLE XXXVI.—INFLUENCE OF CONCENTRATION OF GELATIN PHOSPHATE OF pH OF ABOUT 3.6 ON THE OSMOTIC PRESSURE
(All experiments were made in two sets)

	Concentration of gelatin in per cent									
	2	2	1½	1½	1	1	¾	¾	½	½
pH inside at equilibrium	3.64	3.66	3.60	3.60	3.65	3.66	3.60	3.60	3.61	3.62
pH outside at equilibrium	3.02	3.02	3.02	3.01	3.12	3.11	3.14	3.12	3.21	3.19
y = CH inside $\times 10^5$	22.9	21.9	25.1	25.1	22.4	21.9	25.1	25.1	24.6	24.0
x = CH outside $\times 10^5$	95.5	95.5	95.5	97.7	75.9	77.6	72.4	75.9	61.7	64.6
$z = \frac{(x+y)(x-y)}{y}$	375.0	395.0	338.0	355.0	235.0	253.0	184.0	204.0	130.0	150.0
$2y + z - 2x$	230.0	248.0	197.0	210.0	128.0	142.0	89.0	102.0	56.0	69.0
Observed osmotic pressure	860.0	860.0	715.0	680.0	420.0	445.0	314.0	316.0	186.0	186.0
Calculated osmotic pressure (ignoring gelatin)	576.0	620.0	493.0	523.0	320.0	355.0	222.0	255.0	140.0	172.0
Difference (osmotic pressure due to gelatin)	284.0	240.0	222.0	157.0	100.0	90.0	92.0	61.0	46.0	14.0
Mean	262.0		190.0		95.0		73.0		26.0	

The experiment just described for gelatin phosphate was repeated for gelatin chloride, with similar results.

According to Dakin's[1] recent analyses gelatin contains 1.4 per cent phenylalanine. Since one molecule of gelatin cannot contain less than one molecule of phenylalanine and since the molecular weight of this amino-acid is 165 the lowest possible weight of gelatin is 11,800. If a molecule of gelatin contains two molecules of phenylalanine, the molecular weight should be about 23,600. This would be approximately the figure we might expect from the data of Table XXXVI on the assumption that the differences in the last two rows may be considered to be the values of the osmotic pressure of the protein particles.

TABLE XXXVII.—INFLUENCE OF CONCENTRATION OF ALBUMIN CHLORIDE OF pH OF ABOUT 3.4 ON THE OSMOTIC PRESSURE

	Concentration of egg albumin in per cent					
	4	3	2	1	½	¼
pH inside at equilibrium	3.34	3.32	3.38	3.40	3.40	3.40
pH outside at equilibrium	2.98	2.97	3.07	3.14	3.19	3.24
y = CH inside $\times 10^5$	45.7	47.9	41.7	39.8	39.8	39.8
x = CH outside $\times 10^5$	104.7	107.2	85.1	72.4	64.5	57.5
$z = \frac{(x+y)(x-y)}{y}$	194.0	192.0	132.0	92.0	64.6	43.3
$2y + z - 2x$	76.0	74.0	45.0	27.0	15.0	8.0
Observed osmotic pressure	776.0	555.0 +	375.0	163.0	75.0	36.0
Calculated osmotic pressure (ignoring albumin)	190.0	185.0	113.0	67.0	39.0	20.0
Difference (osmotic pressure due to albumin)	586.0	370.0 +	262.0	96.0	36.0	16.0

A similar experiment was made with different concentrations of solutions of the chloride of crystalline egg albumin. The original pH of the albumin chloride solution was 3.5 and that of the outside solution 3.0. After equilibrium was established the pH both inside and outside was slightly changed as is shown in Table XXXVII. The osmotic pressures for 0.25 to 4 per cent solutions

[1] DAKIN, H. D., *J. Biol. Chem.*, vol. 44, p. 499, 1920.

of albumin chloride were measured and calculated for $2y + z - 2x$. The difference, which should be the osmotic pressure of the albumin particles in solution, is found in the last row. It is almost identical with the difference found for gelatin chloride for the same concentration of gelatin.

We therefore come to the conclusion that the Donnan equilibrium theory allows us to explain and to derive mathematically the influence of pH, of valency of ions, of concentration of neutral salt, and of concentration of protein on the osmotic pressure; and that the values calculated for the osmotic pressure on the basis of this theory agree within the limits of the accuracy of the experiments with those actually observed, though the accuracy of the experiments is considerably less than in the case of the P.D. measurements.

CHAPTER XI

SWELLING

Procter (1914) and Procter and Wilson (1916) applied Donnan's equilibrium theory to the explanation of the swelling of gelatin in acid. According to these authors, the force which causes the entrance of water and hence the increase of volume in a solid block of gelatin in acid is osmotic, and the opposing force which limits the swelling is the force of cohesion between the gelatin molecules or ions constituting the framework inside of which the water is occluded. These cohesive forces thereby play the same role in the swelling equilibrium as does the hydrostatic pressure on the membrane in the experiments on osmotic pressure.

The protein ions constituting a jelly of gelatin chloride cannot diffuse and hence, according to Procter and Wilson, can exercise no measurable osmotic pressure, while the chlorine anions in combination with them are retained in the jelly by the electrostatic attraction of the gelatin ion but exert osmotic pressure. This difference in the diffusibility of the two opposite ions of the gelatin chloride gives rise to the condition leading to the establishment of Donnan's membrane equilibrium. It is immaterial for this equilibrium whether the diffusion of dissolved protein ions is prevented by a collodion membrane, or whether it is prevented by the forces of cohesion between the gelatin ions of a solid gel. If x be the concentration of the H and Cl ions in the outside solution, y the concentration of the free H and Cl ions in the solid gel, and z the concentration of Cl ions in combination with gelatin, the Donnan equilibrium is expressed by the equation

$$x^2 = y(y + z)$$

and the osmotic force e for the absorption of water by the gel is

$$e = 2y + z - 2x$$

The reader will notice that this is the formula applied later by the writer to osmotic pressure.

J. A. and W. H. Wilson[1] developed Procter's line of reasoning further and derived the following formula by purely mathematical reasoning from the assumption that gelatin combines chemically with hydrochloric acid to form a highly ionizable gelatin chloride:

$$V(K + y)(CV + 2\sqrt{CY_y}) - y = 0$$

where V is the increase in volume in cubic centimeters of one milliequivalent weight of gelatin, C is the constant corresponding to the modulus of elasticity of the gelatin, and K is a constant defined by the equation

$$[\text{gelatin}]\,[H^+] = K[\text{gelatin ion}]$$

Given the constants, it is obviously possible to calculate all the variables of the equilibrium.

Procter and Wilson found the value $K = 0.00015$ by means of the hydrogen electrode on gelatin solutions and the value $C = 0.0003$ at 18°C. from experiments on the swelling of gelatin jellies. From Procter's data on gelatin, Wilson[2] calculated 768 as its equivalent weight. Using these constants, Wilson and Wilson calculated the variables V, y, and z for comparison with the data obtained experimentally by Procter. The calculated and observed results are shown in Table XXXVIII and it will be seen that the agreement is absolute, within the limits of Procter's experimental error. This is shown even more strikingly when the values are plotted. Procter and Wilson regard this as establishing their theory quantitatively.

The relation of V to e is governed by Hooke's law, *ut tensio sic vis*, and since e represents a pressure equal in all directions, the result is a pull upon the jelly equal in each dimension. The quantitative expression is

$$e = CV$$

where the constant C is determined by the bulk modulus of the gelatin.

[1] Wilson, J. A., and Wilson, W. H., *J. Am. Chem. Soc.*, vol. 40, p. 886, 1918.

[2] Wilson, J. A., *J. Am. Leather Chem. Assn.*, vol. 12, p. 108, 1917.

TABLE XXXVIII[1]

x	V		y		z	
	Calculated	Observed	Calculated	Observed	Calculated	Observed
0.0032	43.2	41.2	0.0005	0.0005	0.018	0.017
0.0073	40.8	44.5	0.002	0.002	0.022	0.018
0.0077	40.2	40.1	0.002	0.002	0.023	0.020
0.0120	37.5	39.9	0.005	0.006	0.026	0.021
0.0122	37.3	39.7	0.005	0.006	0.026	0.021
0.0170	34.5	31.1	0.008	0.009	0.028	0.028
0.0172	34.3	37.0	0.008	0.009	0.028	0.022
0.0406	26.7	28.0	0.026	0.030	0.037	0.031
0.0420	26.4	23.4	0.027	0.030	0.038	0.038
0.0576	24.0	26.1	0.041	0.043	0.041	0.036
0.0666	23.0	21.4	0.049	0.050	0.043	0.045
0.0680	22.8	22.4	0.050	0.053	0.044	0.039
0.0930	20.7	17.7	0.072	0.072	0.049	0.054
0.0944	20.5	20.3	0.073	0.072	0.049	0.049
0.1052	19.8	22.9	0.083	0.085	0.051	0.043
0.1180	18.9	18.7	0.095	0.090	0.053	0.058
0.1434	17.9	18.4	0.118	0.118	0.056	0.055
0.1435	17.9	18.6	0.118	0.118	0.056	0.054
0.1685	17.1	18.0	0.141	0.138	0.059	0.062
0.1925	16.3	15.8	0.164	0.161	0.061	0.068
0.1940	16.2	17.4	0.166	0.165	0.061	0.060
0.1945	16.2	17.0	0.167	0.164	0.061	0.062

[1] Observed values are taken from PROCTER, H. R., *J. Chem. Soc.*, vol. 105, p. 313, 1914. The observed value for V given in this table is the increase in volume in cubic centimeters of 0.768 gm. of gelatin. Values for x, y, and z are given in moles per liter.

Procter and Wilson then explain on the basis of the Donnan equation why the value of e, and therefore also V, should follow a curve of the particular type it does. By proper substitution from the thermodynamic and osmotic equations it follows that:

$$e = -2x + \sqrt{4x^2 + z^2}$$

"As the concentration of acid is increased from zero to some small, but finite, value, z must necessarily increase at a very much greater rate than x. This is shown very markedly in the most dilute solutions, where almost all the acid added combines with the gelatin: but z has a limiting value, which is determined by the total concentration of gelatin with which we started. Now z must either approach this limiting value or diminish, which it would do if the ionization of the gelatin chloride were sufficiently repressed. In either case:

$$\lim_{x = \infty} \sqrt{4x^2 - x^2} = \sqrt{4x^2},$$

from which it follows that:

$$\lim_{x = \infty} e = -2x + 2x = 0$$

It is clear from this that, as x increases from zero, e must increase to a maximum and then decrease, approaching zero asymptotically, regardless of whether or not the ionization of the gelatin salt is appreciably repressed."[1]

As far as the depressing action of salt on swelling is concerned, Procter and Wilson do not accept the idea that it is due to the repression of ionization.

"Whilst the salt undoubtedly represses the ionisation of the gelatin chloride to some extent, it would scarcely be sufficient to account for the fact that salt reduces the volume of jelly almost to that of dry gelatin. The chief action is probably that the addition of salt corresponds with an increase in the value of x, and that this increase in x must, according to the equation just discussed, produce a decrease in the value of e, with a corresponding diminution of the volume of the jelly."[1]

There can be little doubt that the osmotic theory of Procter and Wilson accounts quantitatively for the process of swelling; no other theory has thus far been offered which can claim the same result.

The force which opposes and limits the swelling is the cohesion between the molecules or ions constituting the gel. When this force is diminished the swelling should increase. Procter and Wilson have pointed out that this is the case since the swelling of gelatin increases when the gel is heated.[2]

The forces of cohesion depend not only on temperature but also on chemical constitution. They are forces of the same kind as the forces determining solution; and it is well known that, *e.g.*, the substitution of Na for H in oleic acid increases the solubility of the substance in water, and that the substitution of K for Na increases the solubility still more. We might *a priori* expect that the forces of cohesion in a solid jelly of gelatin would also change considerably with the nature of the ion in combination with

[1] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, vol. 109, p. 317, 1916.
[2] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, vol. 109, p. 315, 1916.

the gelatin. This is, however, as a rule, not the case. Only the valency but not the nature of the ion in combination with gelatin influences the swelling of gelatin. Thus, at the same temperature, at the same pH, and the same concentration of originally isoelectric gelatin, the swelling of gelatin chloride, nitrate, trichloracetate, oxalate, tartrate, phosphate, citrate, etc., is approximately the same, while that of gelatin sulphate is considerably lower. The swelling of Li, Na, K, and NH_4 gelatinate is also practically the same at the same pH and the same concentration of originally isoelectric gelatin, but the swelling of Mg, Ca, and Ba gelatinate is considerably less (see Chap. V).

It was shown in Chap. V that the same valency effect which exists in regard to osmotic pressure exists also in regard to swelling, and the theoretical discussion given in the preceding chapter for this valency effect in the case of osmotic pressure covers also the similar effect in the case of swelling.

In the case of casein-acid salts, which are less soluble than gelatin-acid salts, the nature of the anion is not without influence on the cohesive forces. Thus casein trichloracetate is practically as insoluble as casein sulphate, and neither of the two salts is capable of swelling; while the more soluble casein chloride and casein phosphate are capable of swelling. In the latter case the valency rule also holds since the degree of swelling is practically the same for casein phosphate and casein chloride, at the same pH temperature and concentration of originally isoelectric casein.[1] The valency rule holds wherever colloidal behavior is concerned, since colloidal behavior is only the consequence of the Donnan equilibrium and the equilibrium equation is only concerned with the sign and valency of the ion. The problems of solubility and of cohesion have only an indirect connection with colloidal behavior, and the fact that solubility and cohesion depend upon the specific nature of the ion (in addition to its sign of charge and valency) is not in conflict with the other fact that in the truly colloidal phenomena only the sign of charge and valency of an ion are concerned.

At the isoelectric point gelatin is practically not ionized and there can therefore be no Donnan equilibrium. Yet when dry

[1] Loeb, J., and Loeb, R. F., *J. Gen. Physiol.*, vol. 4, p. 187, 1921–22.

grains of isolectric gelatin are put into water of pH 4.7, a considerable swelling occurs. The swelling must be determined by forces different from those set up by the Donnan equilibrium. In the first place, there are those forces of chemical attraction between the molecules of water and certain of the groups of the gelatin molecule which cause the solution of gelatin in water when the forces of cohesion between the gelatin molecules forming the gel can be overcome. The absorption of water by dry grains of isoelectric gelatin at pH 4.7 is, therefore, primarily but in all probability not exclusively due to the residual valency forces, and the swelling of solid isoelectric gelatin granules is primarily a phenomenon of solid solution.

CHAPTER XII

VISCOSITY[1]

1. We have seen in Chaps. V and VI that the influence of electrolytes on the viscosity of the solutions of certain proteins, *e.g.*, gelatin or casein, is similar to the influence of electrolytes on osmotic pressure, swelling, and potential differences. The explanation given for the influence of electrolytes on the last named properties was based on the theory of Donnan's membrane equilibirum. This theory can only be applied where the diffusion of one type of ions is prevented, while no such block exists for other ions. In the experiments on osmotic pressure or P.D. of protein solutions the collodion membrane permits the diffusion of crystalloidal ions while preventing the diffusion of the protein ions; and in the case of the solid gel the protein ions are prevented by the forces of cohesion from diffusing into the surrounding solution free from protein. But this raises the problem of how the Donnan equilibrium can be applied to the viscosity of protein solutions. We intend to show that the answer lies in the fact that although protein solutions may be and probably are as a rule true solutions, consisting of isolated protein ions and molecules distributed equally through the water, they contain under certain conditions submicroscopic solid particles of protein. We shall see that the viscosity of protein solutions is only influenced in the same way by electrolytes as is the osmotic pressure, when such solid protein particles are present in considerable numbers. If they are absent, or if they are scarce, electrolytes will not influence the viscosity of protein solutions in the same way as electrolytes influence the osmotic pressure or the P.D. of protein solutions. In the following discussion we shall measure the viscosity of protein solutions by the time of outflow through a capillary tube, as described by Ostwald, and the quotient of this time over the time of outflow of pure water through the same viscometer at

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 827, 1920–21; vol. 4, pp. 73, 97, 1921–22. Loeb, J., and Loeb, R. F., *J. Gen. Physiol.*, vol. 4, p. 187, 1921–22.

the same temperature will be referred to as the relative viscosity or as the viscosity ratio of the protein solution. This method of measuring the relative viscosity will require improvement but it suffices for an approximate test of the validity of the theory.

Einstein[1] has developed a theory of the viscosity of solutions which makes the viscosity a linear function of the relative volume occupied by the solute in the solution

$$\eta = \eta_0(1 + 2.5\varphi) \tag{1}$$

where η_0 is the viscosity of the water at the temperature of the experiment, η the viscosity of the solution, and φ the fraction of the volume occupied by the solute in the volume of the solution. As Einstein points out, this formula can only be used when φ is very small and when the particles of the solute are spherical and large in comparison with the molecules of the solvent. This condition is no longer fulfilled in protein solutions when the relative volume occupied by the protein in the solution becomes too large.

Several authors have tried to modify Einstein's formula in order to make it applicable to higher concentrations of protein solutions. Hatschek[2] proposed to replace the constant 2.5 of Einstein's formula by the constant 4.5, but his deductions have been criticised both by Smoluchowski[3] and by Arrhenius.[4] Arrhenius has shown that a logarithmic formula, which he derives very ingeniously from Einstein's formula, fits the actual observations in a satisfactory way, this formula being

$$\log \eta - \log \eta_0 = \vartheta\varphi \tag{2}$$

where φ is again the fraction of volume occupied by the protein in solution, ϑ a constant, while η and η_0 have the same significance as in Einstein's equation. We shall make use of Arrhenius's equation (2) when we are dealing with higher viscosities.

Both the formulæ of Einstein and of Arrhenius make the viscosity a function of the relative volume occupied by the solute

[1] Einstein, A., *Ann. Physik*, vol. 19, p. 289, 1906: vol. 34, p. 591, 1911.
[2] Hatschek, E., *Kolloid-Z.*, vol. 11, p. 280, 1912.
[3] Smoluchowski, M., *Kolloid-Z.*, vol. 18, p. 190, 1916.
[4] Arrhenius, S., *Meddelanden K. Vetenskapsakademiens Nobelinstitut*, vol. 3, No. 21, 1917.

in the solution, and it must be our task to correlate the influence of electrolytes on viscosity with corresponding variations of the volume of the protein in solution.

The question then arises, How can the same mass of protein particles in solution change its relative volume under the influence of electrolytes? This is only possible if the relative volume occupied by the protein in the solution is increased by water shifting from solvent to solute. We, therefore, have to find out whether or not a shifting of water from the solvent to the solute is possible, so that the volume of the solvent is diminished and that of the solute increased. It is generally assumed that the mechanism for such a transfer of water from solvent to solute is explained by Pauli's hydration theory which has been repeatedly referred to in this volume. Pauli suggested that the ionized molecule of protein is surrounded by a shell of water which is lacking in the non-ionized molecule. When protein is ionized, *i.e.*, by the addition of acid or alkali to isoelectric protein, a shell of water is formed around each individual protein ion. On this basis we can understand why the viscosity of a solution of isoelectric protein should increase with the addition of acid or alkali. The work of Lorenz, Born, and others, however, casts a doubt on the assumption of a general hydration of polyatomic ions. There are still other facts which show that the mere ionization and consequent hydration of the individual protein ions cannot well be the cause of the influence of the pH on the relative viscosity of gelatin solutions.

Gelatin solutions show the characteristic influence of the pH on their viscosity, as is demonstrated in Fig. 48. The viscosity of gelatin solutions behaves qualitatively as we might expect on the basis of Pauli's hydration theory. Yet, if hydration of the individual protein ions were the cause of the variation of the viscosity of gelatin solutions, a variation of the hydrogen ion concentration should have a similar influence on the viscosity of solutions of simple amino-acids, like glycocoll and alanine, to that which it has on the viscosity of gelatin solutions. Five per cent solutions of glycocoll and alanine were brought to different pH, from 5.0 to 2.0 and below, by the addition of HCl. Miss Brakeley found, in the writer's laboratory, that the variation of the pH of 5 per cent solutions of these two amino-acids between

the limits of 5.0 and 1.16 had no measurable influence on the viscosity of the solution. G. Hedestrand[1] found in Euler's laboratory a slight variation in the viscosity of 2 N glycocoll solutions upon the addition of acid or alkali; the minimum was found at pH 6.4 where the viscosity was about 1.36, while at

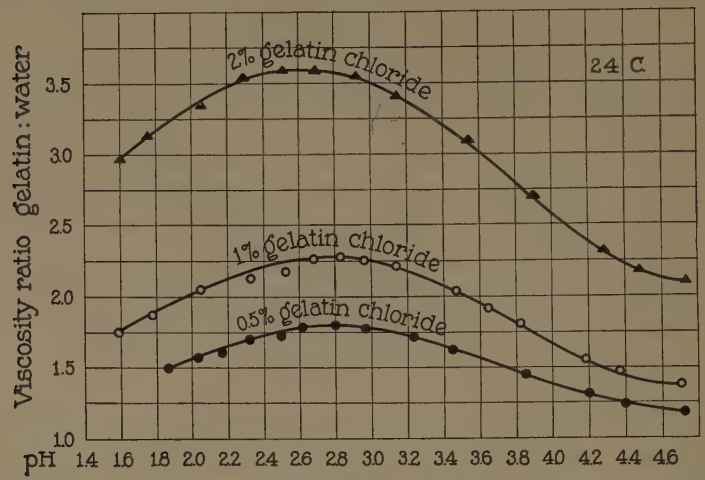

FIG. 48.—Influence of pH on viscosity of freshly prepared gelatin chloride solutions.

pH 3.0 it was 1.38. This is an influence of pH of a much lower order of magnitude than the one found in the case of gelatin solutions or casein solutions. These results cast a serious doubt on the assumption that the variations in the curve of the viscosity of gelatin, as expressed in Fig. 48, were caused by variations in the hydration of the individual gelatin ions.

This doubt was increased by experiments on the influence of pH on the viscosity of crystalline egg albumin which indicated only a slight, almost negligible influence of the pH on the viscosity. Figure 49 gives such an experiment with 3 per cent originally isoelectric albumin brought to different pH through the addition of HCl. The ordinates are the viscosity ratios of albumin solution over water, drawn on a larger scale than those in Fig. 48, and the abscissæ are the pH of the solution. It is obvious that if compared with the gelatin curves the pH has only a very slight

[1] HEDESTRAND G., *Arkiv Kemi, Min. och Geol.*, vol. 8, p. 1, 1921.

influence on the viscosity of solutions of crystalline egg albumin between pH 4.6 and pH 1.0. With a further lowering of pH the viscosity suddenly rises, a fact to which we shall return later.

The method of the experiments was as follows: 50-c.c. samples of a 6 per cent solution of isoelectric crystalline egg albumin were mixed with 50 c.c. of HCl solutions of different concentrations and the pH measured. The solutions were rapidly brought to a temperature of 24°C. and the viscosity was measured immediately at that temperature.

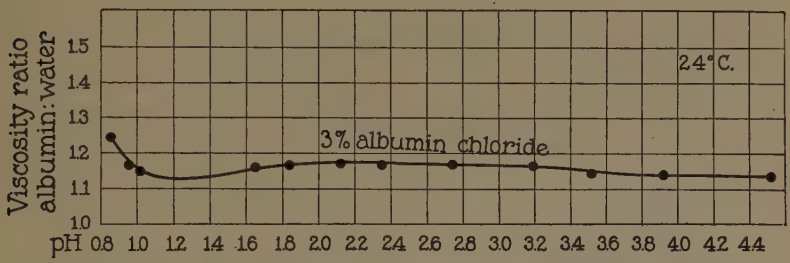

FIG. 49.—Showing that solutions of crystalline egg albumin have a low viscosity in comparison with gelatin solutions, and that the pH has little influence on the viscosity of solutions of crystalline egg albumin at pH over 1.0 and at ordinary temperature.

The question then arises, Why do amino-acids and at least one protein, namely crystalline egg albumin, behave so differently from gelatin in regard to the influence of the pH on the viscosity of their solutions? As long as we assume that the influence of the hydrogen ion concentration on the viscosity of gelatin-acid salt solution is due to the hydration of the individual protein ions this difference is incomprehensible, since the amino-acids as well as crystalline egg albumin should in this case show the same influence of ionization on viscosity as the gelatin.

The puzzle becomes still greater if we take into consideration the fact that the osmotic pressure of solutions of crystalline egg albumin is affected in the same way by the hydrogen ion concentration as is the osmotic pressure of gelatin solutions (Chap. V). Why then do these two proteins behave so differently as regards the influence of the pH on their viscosity?

We get an answer to this question by comparing the order of magnitude of the viscosity of solutions of crystalline egg albumin and of gelatin. The viscosity of solutions of crystalline egg

albumin has a comparatively low order of magnitude if compared with the viscosity of solutions of gelatin of the same concentration of protein and the same pH. The viscosity of solutions of crystalline egg albumin of pH 5.1, (*i.e.*, near the isoelectric point) of concentrations from 1 to 14 per cent, was measured at 15°C. (Fig. 50). The viscosity is not only low but is also practically a linear function of the concentration. Figure 51 gives the viscosity of different concentrations of solutions of

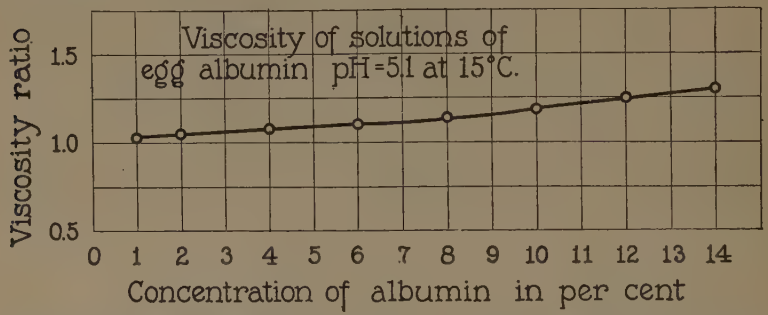
Viscosity of solutions of
egg albumin pH=5.1 at 15°C.
Viscosity ratio
1.5
1.0
0.5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Concentration of albumin in per cent

FIG. 50.—Viscosity ratio of solutions of crystalline egg albumin near the isoelectric point. Inside the concentrations used, the viscosity ratio is nearly a linear function of the concentration.

isoelectric gelatin at different temperatures. The solutions were prepared from the same stock solution of isoelectric gelatin and were rapidly heated to 45°C. and rapidly cooled to the desired temperature and then the time of outflow in an Ostwald viscometer was measured. This was done to avoid the increase in viscosity which occurs on standing and which is especially noticeable in the case of solutions of isoelectric gelatin. For the sake of conformity the same procedure was followed in the case of solutions of crystalline egg albumin. It is obvious that where the pH influences the viscosity in the same sense as the osmotic pressure, *e.g.*, in the case of gelatin solutions, the viscosity is of a much higher order of magnitude than where the pH has no such influence on viscosity as is the case in solutions of crystalline egg albumin.

It now remains to show that this difference in the order of magnitude of the viscosity of the two solutions is connected with the relative volume occupied by the protein in solution. The low order of magnitude of the viscosity of solutions of crystalline

egg albumin suggests a small relative volume; and if this be true the viscosity of solutions of crystalline egg albumin should obey the Einstein formula; while the high order of magnitude of viscosity of the solutions of gelatin suggests that a larger volume is

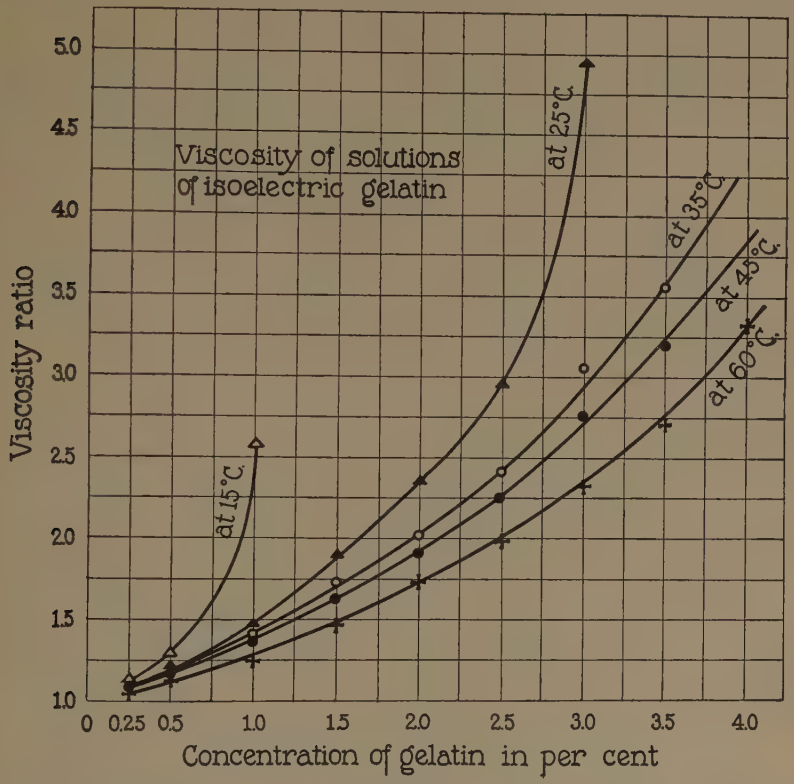

FIG. 51.—Influence of concentration on the viscosity of solutions of isoelectric gelatin.

occupied by the gelatin particles in solution and hence the constant 2.5 of Einstein's formula should be found too small; in other words, the Einstein formula should be replaced by some other formula, *e.g.*, that of Arrhenius.

Einstein's formula is $\frac{\eta}{\eta_0} = 1 + 2.5\varphi$, where φ is the relative volume occupied by the protein in the solution, and $\frac{\eta}{\eta_0}$ is the

viscosity ratio, *i.e.*, time of outflow of solution over time of outflow of water. The volume occupied by the protein in 100 c.c. of solution is

$$\varphi = \left(\frac{\eta}{\eta_0} - 1\right)\frac{100}{2.5}$$

By dividing the weight of albumin in solution by its volume we should obtain the density of albumin. Determinations of the density of albumin, by direct methods, give the value of 1.36 (Arrhenius). Table XXXIX shows that if we calculate the

TABLE XXXIX

Concentration of crystalline egg albumin, per cent	$\frac{\eta}{\eta_0} - 1$	Calculated volume of albumin, cubic centimeters	Calculated density of albumin
14	0.290	11.6	1.20
12	0.240	9.6	1.25
10	0.185	7.4	1.35
8	0.132	5.3	1.51
6	0.100	4.0	1.50
4	0.074	2.96	1.36
2	0.042	1.7	1.17

density of albumin on the basis of Einstein's formula, we obtain values which differ only inside the limits of accuracy from the value 1.36 obtained by direct determination. The time of outflow of water through the viscometer was in this case 227 seconds at 15°C. These measurements show that the low order of magnitude of the viscosity of solutions of crystalline egg albumin is accompanied by a volume of albumin sufficiently low to permit the application of Einstein's formula, with the constant 2.5.

When we try to apply Einstein's formula in the same way to the viscosity measurements of isoelectric gelatin solutions we find that the relative volume of gelatin in the solution and its density calculated on the basis of the constant 2.5 lead to impossible results. Thus the density of gelatin is probably not very differ-

ent from that of egg albumin, *i.e.*, in the neighborhood of 1.4. The values calculated in Table XL with Einstein's viscosity constant 2.5 are from 20 to 40 times too low. Hence, the relative

TABLE XL

Concentration of isoelectric gelatin, per cent	$\frac{\eta}{\eta_0} - 1$ at 35°	Calculated volume of gelatin, cubic centimeters	Calculated density of gelatin
0.5	0.170	6.8	0.077
1.0	0.405	16.2	0.06
1.5	0.725	29.0	0.05
2.0	1.020	40.8	0.05
2.5	1.405	56.2	0.045
3.0	2.042	81.7	0.037
3.5	2.560	102.4	0.034

volume of gelatin in these solutions is far beyond the limit inside which the formula of Einstein is applicable. The formula of Arrhenius (2) leads to a fair agreement. According to this formula the logarithms of the viscosity ratio when plotted over the concentration of the gelatin should give a straight line. The agreement of the values for 45 and 35° with this theory is satisfactory (considering the limits of accuracy of the measurements) the logarithms of the viscosity increasing practically in direct proportion with the concentration (*i.e.*, the relative volume) of the gelatin in the solution (Table XLI). At 60° the agreement is not quite so good but still recognizable. At 25°C., however, it is satisfactory only at the lowest concentrations, but at the higher concentrations the viscosity grows more rapidly than the concentration. The reason for this is, however, obvious, since at this temperature the gelatin solution solidifies so rapidly that the viscosity measurements were no longer possible for a concentration of 3.5 per cent gelatin solution, and for this reason the value of the viscosity of a 3 or a 2 per cent solution is already too high on account of the mechanical hindrance of the flow of the solution through the viscometer owing to partial solidification.

TABLE XLI

Concentration of solution of isoelectric gelatin, per cent	$\log \frac{\eta}{\eta_0}$			
	60°	45°	35°	25°
0.25	0.0236	0.0306	0.0269	0.0374
0.5	0.0504	0.0682	0.0682	0.0792
1.0	0.0930	0.1350	0.1475	0.1685
1.5	0.1656	0.2135	0.2367	0.2765
2.0	0.2350	0.2796	0.3057	0.3701
2.5	0.2953	0.3512	0.3811	0.4691
3.0	0.3094	0.4409	0.4832	0.6941
3.5	0.4321	0.5051	0.5514	solidifies
4.0	0.5214	0.5660	0.6043	

These experiments lead to the following two conclusions.

(*a*) Since the viscosity measurements of solutions of crystalline egg albumin and of gelatin agree fairly well with Einstein's and Arrhenius's formula respectively, it seems that the viscosity of the solutions of proteins is primarily a function of the relative volume occupied by the protein in solution.

(*b*) Since the measurements were made at (or near) the isoelectric point of the two proteins the difference in the viscosity of solutions of gelatin and of crystalline egg albumin cannot be ascribed to differences in the degree of hydration of the individual protein ions, since at the isoelectric point the protein is not ionized.

It follows from these results, that the difference in the order of magnitude of the viscosity of the two proteins must be due to the fact that gelatin possesses a mechanism for increasing its relative volume in solution which is lacking in the case of egg albumin (in not too high a concentration, at not too high a temperature and a pH above 1.0), and this mechanism seems to be connected, in the case of gelatin solutions, with their tendency to set to a gel.

Zsigmondy (p. 98) states that Smoluchowski has explained the increase in the viscosity of a solution of aluminium oxide upon coagulation by the assumption of an occlusion of liquid between the particles. Smoluchowski calculates from the

increase of viscosity during coagulation of aluminium oxide that the coagulating particles occupy a volume 400 to 500 times as great as that occupied by the dry material itself.[1] This apparent increase of volume he explains through the aggregation of needle-shaped particles, water being occluded between these particles. Smoluchowski apparently did not associate this occlusion of water with the Donnan equilibrium.

If we adopt this idea for the explanation of the high order of viscosity of gelatin solutions as compared with solutions of egg albumin we reach, the conclusion that the gelatin solutions contain submicroscopic particles of solid jelly, *i.e.*, micellæ which occlude relatively large quantities of water, whereby the relative volume occupied by the gelatin in solution is increased, and that such particles are lacking or scarce in the case of solutions of egg albumin. These particles of solid jelly are the precursors of the continuous jelly to which the gelatin solution has a tendency to set. The fact that these particles are lacking or scarce in the case of solutions of egg albumin is connected with the fact that the latter solutions have no tendency to set to a jelly at ordinary temperature and a pH above 1.0. When the pH is below 1.0 and the temperature higher the solutions of crystalline egg albumin set to a jelly and in that case their viscosity becomes of the same high order of magnitude as that of gelatin solutions.

This assumption would also explain why the pH causes a similar variation in the viscosity of gelatin solutions as in their osmotic pressure, while the viscosity of solutions of crystalline egg albumin shows no such influence of the pH. There must arise a Donnan equilibrium between these submicroscopic particles of solid jelly and the surrounding solution, and this Donnan equilibrium must regulate the amount of water occluded by the submicroscopic particles of solid jelly, floating in the gelatin solution. Since the low order of magnitude of the viscosity of albumin solutions excludes the existence of a considerable number of such submicroscopic solid particles in the solution, it becomes obvious that the Donnan equilibrium cannot manifest itself to any large extent in the viscosity of solutions of this

[1] Quoted from Zsigmondy. The paper of Smoluchowski is inaccessible to the writer since the number of the journal in which it appeared failed to reach the Institute during and since the war.

protein at not too high a concentration, at low temperatures, and at pH above 1.0.

2. If this assumption is correct, it would follow that a suspension of powdered gelatin in water should have a greater viscosity at a given temperature than if the same mass of gelatin is dissolved in water, since in the latter case part of the gelatin at least is in true solution (as we shall see later) and this latter is incapable of increasing its volume by occluding water. It would follow, furthermore, that the influence of electrolytes on the viscosity of suspensions of powdered gelatin would be the same as the influence of electrolytes on the osmotic pressure of gelatin solutions. It can be shown that both expectations are fulfilled.

Doses of 0.5 gm. of powdered gelatin were put into 100 c.c. of water containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12.5, 15 and 20 c.c. of 0.1 N HCl to bring the gelatin to different pH. The suspensions were allowed to stand 1 hour at 20° to bring about the swelling of the particles, and the viscosity of the suspensions was measured in a straight viscometer at 20°C. The time of outflow of water through the viscometer at 20° was 48.5 seconds. The upper curve in Fig. 52 gives the ratio of viscosity of suspensions to that of water at 20°C. (When the viscosity is high, the values obtained are a little too great owing to a gravity effect which causes the solid particles to collect above the upper opening of the capillary tube during a part of the time of the experiment thus increasing temporarily the density of the suspension.) After the viscosity of a suspension was measured the suspension was transformed into a solution by heating the suspension to 45°C. for 10 minutes; after that the solution was rapidly cooled to 20°C. and the viscosity of the gelatin solution was immediately measured with the same viscometer at 20°C. The lower curve in Fig. 52 shows that the viscosity was now considerably diminished. The abscissæ are the pH of the gelatin solutions.

If we measure the volume of the suspended particles we find that it varies in a similar way as the viscosity. Samples of 0.5 gm. of Cooper's powdered commercial gelatin of a pH of about 6.0 were added to 100-c.c. portions of water containing varying amounts of HCl. The particles had uniform size (going through sieve 100 but not through sieve 120), but their shape was extremely irregular. They were left in the solution several hours

at 20°C., and then their time of outflow through a capillary tube was ascertained at 20°C. The time of outflow of water through the viscometer at this temperature was 24 seconds. It was essential to stir the suspension thoroughly before sucking it into the viscometer since the gelatin particles sink rapidly to the bottom of the dish.

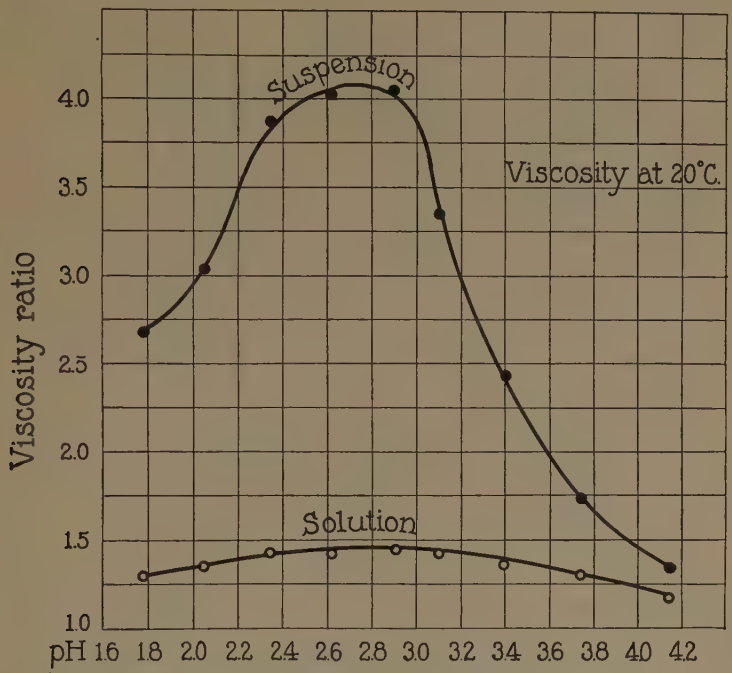

FIG. 52.—Difference in the viscosity of a suspension of 0.5 gm. of powdered gelatin in 100 c.c. and of the solution of the suspension in the same liquid; both viscosities were measured at 20°C.

After the viscosity measurements were taken, the suspension was put on a filter of cotton wool and the supernatant water allowed to drain off. By measuring the volume of the filtrate and deducting this from the original volume of the suspension (which was in all cases 100 c.c.), the volume of the gelatin was obtained (with a considerable error). Then the gelatin was melted and the pH of the melted mass of gelatin as well as of the filtrate was determined potentiometrically. Figure 53 gives the result of such an experiment. The lower curve shows the influence of

the pH (of the gelatin) on the viscosity, and the upper curve the influence of the pH on the volume of the gelatin. The two curves are similar.

The valency of the anion of the acid influences the viscosity of suspensions of protein in a similar way as it does the viscosity of solutions. This proof is furnished in Fig. 54. Doses of 0.5 gm. of finely powdered gelatin (going through a sieve of mesh

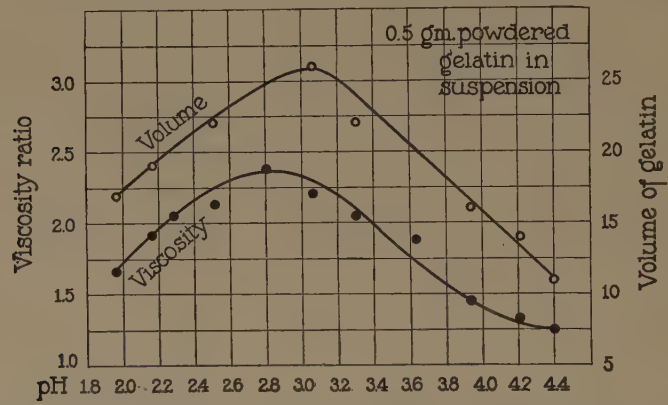

FIG. 53.—Showing that the influence of pH on viscosity of 0.5 per cent suspensions of powdered gelatin in water is similar to the influence of pH on viscosity of gelatin solutions, and that the volume occupied by the particles in the suspension varies in a similar way as the viscosity. Temperature 20°C.

size 100 but not through sieve of mesh size 120) of pH 7.0 were put into a series of beakers containing each 100 c.c. of HCl of different pH and kept in the solution over night at a temperature of 20°C. Simultaneously a similar series of beakers containing each 100 c.c. of H_3PO_4 and H_2SO_4 of different pH (instead of HCl) were prepared, each receiving also 0.5 gm. of powdered gelatin. After 19 hours the viscosities of all these series of suspensions were determined at 20°C. Figure 54 gives the result, the ordinates being the values for the viscosity ratios, gelatin suspension: water, and the abscissæ are the pH of the gelatin particles at equilibrium. The curves show that the viscosity of suspensions of gelatin sulphate is a little less than half that of suspensions of gelatin chloride and phosphate of the same pH. The curves for the suspensions of gelatin chloride and gelatin phosphate are alike, with the exception of part of the descending branch.

Experiments on the influence of these three acids on swelling (Fig. 19, Chap. V) show that the curves for the relative volume of powdered gelatin in solutions of these three acids are similar to the viscosity curves in Fig. 54 since the relative volume of gelatin sulphate was found to be not far from one-half of that of gelatin chloride or gelatin phosphate of the same pH.

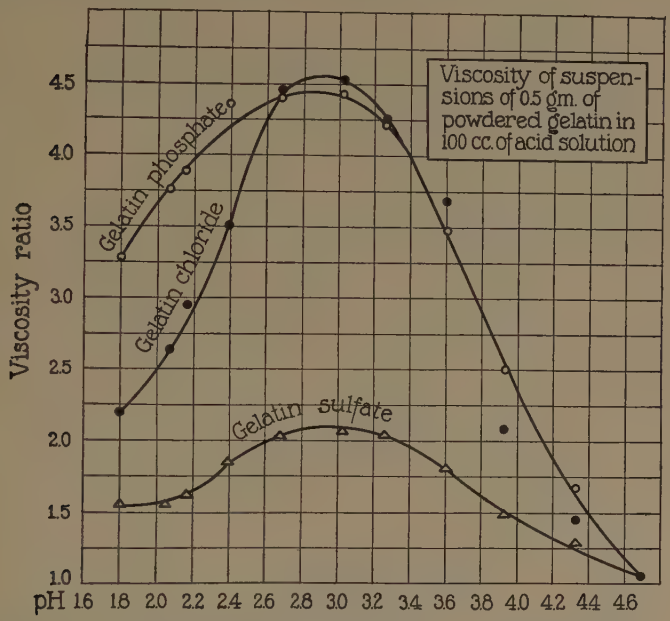

FIG. 54.—Viscosity of suspensions of 0.5 gm. of powdered gelatin of grain size 100 to 120. Abscissæ are the pH, the ordinates the ratio of time of outflow of suspension to time of outflow of water. The influence of HCl and H_3PO_4 is practically identical for the same pH while H_2SO_4 depresses the viscosity of the suspensions to a little less than one-half of that for HCl.

We have seen that the viscosity of a gelatin chloride solution, *e.g.*, of pH 3.0, is lowered when neutral salts are added and the pH kept constant (Fig. 29, Chap. VI). The same is true for the viscosity of suspensions of powdered gelatin. Doses of 0.5 gm. of powdered gelatin of pH 6.0, going through sieve 100 but not through sieve 120, were put each into 100 c.c. of water containing 6 c.c. of 0.1 N HCl, and different quantities of $NaNO_3$, so that the concentration of the salt varied in the different solutions from M/8 to M/2,048. One solution contained no salt. The

pH of the gelatin varied in the neighborhood of 3.0; the temperature was 20°C. After 2½ hours, when the Donnan equilibrium between the particles and the surrounding solution was supposed to be established, the viscosity of each suspension was measured at 20°C. and the volume occupied by the suspended particles of gelatin was ascertained in the manner described. It was found

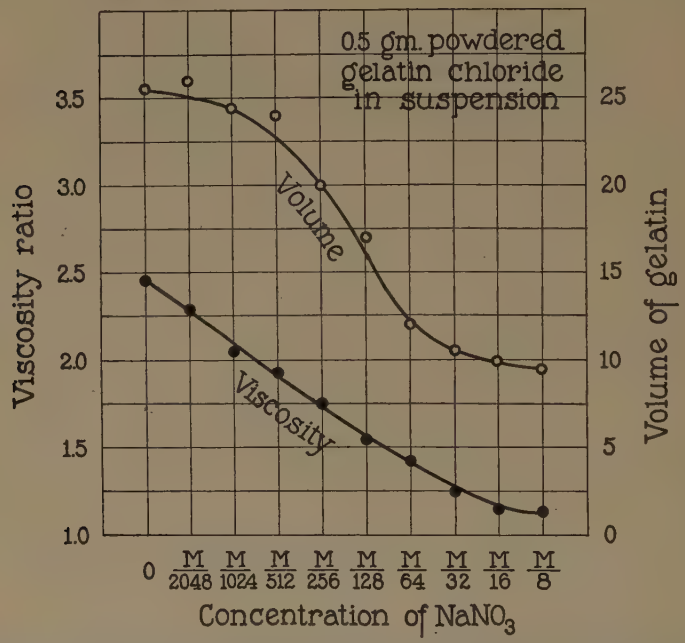

FIG. 55.—Showing depressing influence of neutral salts on viscosity of suspensions of powdered gelatin in water and on the volume occupied by the gelatin particles in the suspension.

that the addition of salt diminished the relative volume of the gelatin particles and the viscosity (Fig. 55). Where the volume of the gelatin was great it no longer varied parallel with the viscosity, as was to be expected from the fact that Einstein's formula no longer holds in this case.

The measurements of the pH of the gelatin solution and the outside solution showed that the addition of salt diminished the difference between the two, as Donnan's theory demands (Table XLII).

TABLE XLII

	Concentration of $NaNO_3$								
	0	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16
pH of gelatin particles	3.04	3.04	3.03	3.02	3.00	3.02	2.97	2.94	2.85
pH of supernatant liquid	2.74	2.76	2.76	2.76	2.77	2.80	2.78	2.77	2.70
Difference, pH inside minus pH outside	0.30	0.28	0.27	0.26	0.23	0.22	0.19	0.17	0.15

This demonstration completes the proof that the viscosity of *suspensions* of powdered gelatin in water of different pH is influenced in the same way by electrolytes as is the viscosity of *solutions* of the same gelatin salts, and that this influence is due, in the case of suspensions, to the influence of the Donnan equilibrium upon the swelling of the particles.

The volume V of gelatin occupied in 100 c.c. of the suspension was determined by filtering and deducting the volume of the filtrate from the total volume of the suspension. Knowing the viscosity we can calculate Einstein's constant c according to the formula

$$\left(\frac{\eta}{\eta_0} - 1\right)\frac{100}{V} = c$$

c should be 2.5 if V is sufficiently small.

TABLE XLIII

V, cubic centimeters	$\frac{\eta}{\eta_0}$	c
12.0	1.292	2.5
17.0	1.480	2.8
18.0	1.792	4.4
20.5	2.064	5.1
20.5	2.020	4.9
20.0	1.855	4.2
18.0	1.625	3.5
16.5	1.542	3.3

The values in Table XLIII show that Einstein's formula gives the correct values for viscosity when the volume of the gelatin is small, since in that case c is equal or nearly equal to 2.5, as his formula demands.

When, however, the volume is larger, the value for c exceeds 2.5. The fact that the value for c exceeds 2.5 when the relative volume occupied by the particles in the solution is large, was found also by Hatschek, Smoluchowski, and Arrhenius. Hatschek replaced the value 2.5 in Einstein's formula by a larger one, namely 4.5. This, however, meets in our case with the difficulty that the value c shows a drift reaching a maximum when the volume of the gelatin particles is a maximum. This difficulty is largely avoided in Arrhenius's formula and we have to change from Einstein's formula to that of Arrhenius whenever the relative volume of the particles in solution or suspension exceeds the limits of the applicability of Einstein's formula, as we shall see in the next chapter.

The experiments on the viscosity of suspensions of powdered gelatin in water have, therefore, led to the result, first, that the influence of pH, of the valency of ions, and of the concentration of neutral salts on the viscosity of *suspensions* of finely powdered gelatin in water is similar to the influence of these three agencies on the viscosity of gelatin *solutions;* second, that the influence of electrolytes on the viscosity of the suspensions is due to the variation of the swelling (or relative volume) of the suspended particles; and third, that this latter fact explains why the Donnan equilibrium determines also the variation of viscosity of these suspensions. If it could be shown that a solution of gelatin contains also some (submicroscopic) particles of solid jelly (capable of swelling), we should understand at once why electrolytes influence the viscosity of gelatin solutions as they influence the swelling, osmotic pressure, or the P.D. of these solutions.

3. We have only indirect means of testing the occlusion theory for gelatin solutions but these tests give an unequivocal answer. When a 0.5 per cent solution of isoelectric gelatin is heated rapidly to 45°, cooled rapidly to a lower temperature, *e.g.*, 20°C., and kept at this temperature, the solution will ultimately set to a continuous gel but will steadily increase its viscosity before this stage is reached. It is natural to assume that the formation

of a continuous jelly is preceded by the formation of submicroscopic pieces of jelly, which increase in number and size, forming finally a continuous jelly. Hence, the longer a solution of isoelectric gelatin stands at 20°C. the greater the number of submicroscopic solid pieces of jelly formed in the solution. The submicroscopic pieces of jelly, surrounded by a true solution of

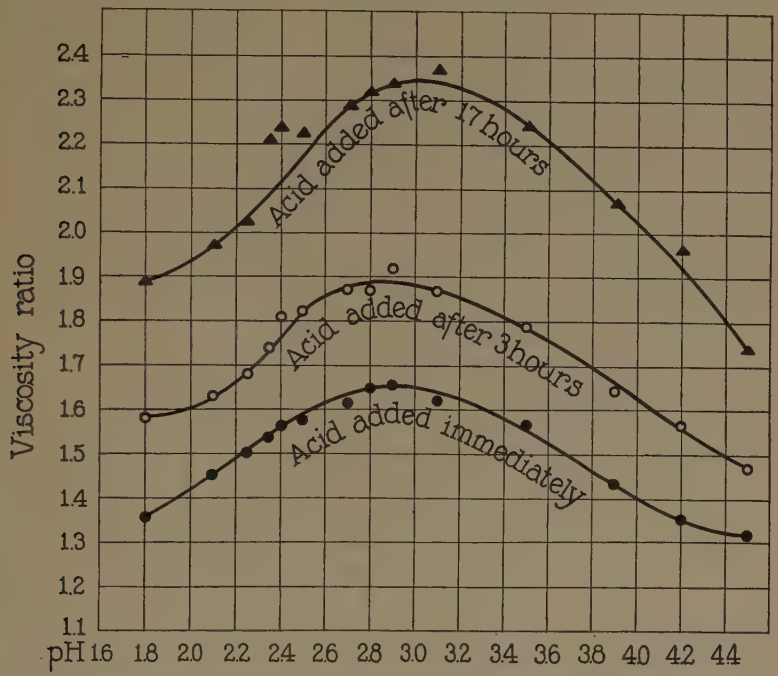

FIG. 56.—Increase in viscosity when acid is added to solutions of isoelectric gelatin after they had been standing for 3 and 17 hours respectively.

isolated molecules of gelatin in water, are compelled to regulate the amount of water they occlude by the Donnan equilibrium. Hence, when we add some HCl to a 0.5 per cent solution of isoelectric gelatin after the solution has been standing for some hours at 20° we should expect to find a higher viscosity than when we add the same amount of acid to the gelatin solution immediately after it has been rapidly heated to 45° and rapidly cooled to 20°C.

This experiment turns out as expected, as is shown in Fig. 56.

When a 0.5 per cent solution of isoelectric gelatin is rapidly heated to 45°C., cooled rapidly to 20°C., and brought immediately to a different pH by the addition of HCl, at 20°C. a viscosity curve like the lowest in Fig. 56 is obtained. When, however, the 0.5 per cent isoelectric gelatin solution is allowed to stand for 3 hours at 20°C. before the acid is added, a parallel viscosity curve is formed at 20°C. but higher than the first one (middle curve, Fig. 56), for the reason that during the 3 hours an additional number of solid jelly particles capable of swelling has been formed. If the solution of isoelectric gelatin stands for 17 hours at 20°C., before the HCl is added, the curve is still higher though practically parallel with the first curve (upper curve, Fig. 56), except at the summit. It is probable that on standing not only the number but the size of individual particles also increases, and the writer has observed that for the size of granules used in his experiments the greater the size the greater the viscosity, since the viscosity is chiefly but not exclusively a function of the relative volume of the particles.

Since jelly formation of gelatin is a reversible process we should expect that two opposite processes always take place simultaneously in a gelatin solution on standing, namely, first, the formation of solid particles of jelly through the aggregation of previously isolated gelatin molecules and ions, and second, the dissolution of such aggregates (micellæ) back into isolated molecules and ions. It is easy to show that powdered gelatin of a given pH dissolves the more rapidly the higher the temperature. If, therefore, our assumption is correct that in a solution of gelatin two opposite processes go on constantly, the rate of melting of the micellæ should increase if the temperature rises. Hence, at very low temperature the viscosity of a gelatin solution should increase rapidly on standing since the formation of new micellæ takes place constantly, while practically no melting of micellæ occurs. When, however, the temperature is raised beyond a certain point, the rate of melting of micellæ increases more rapidly than the rate of formation of new micellæ. Hence, at such a temperature the viscosity of a gelatin solution should not increase but decrease on standing.

This conclusion was tested experimentally and found to be correct. A 2 per cent solution of gelatin chloride of pH 2.7 was

rapidly heated to a temperature of 45° and then rapidly brought to the temperature at which the change of viscosity of the solution with time was to be observed. At definite intervals the viscosity of the solution was measured. Figure 57 gives the result. At

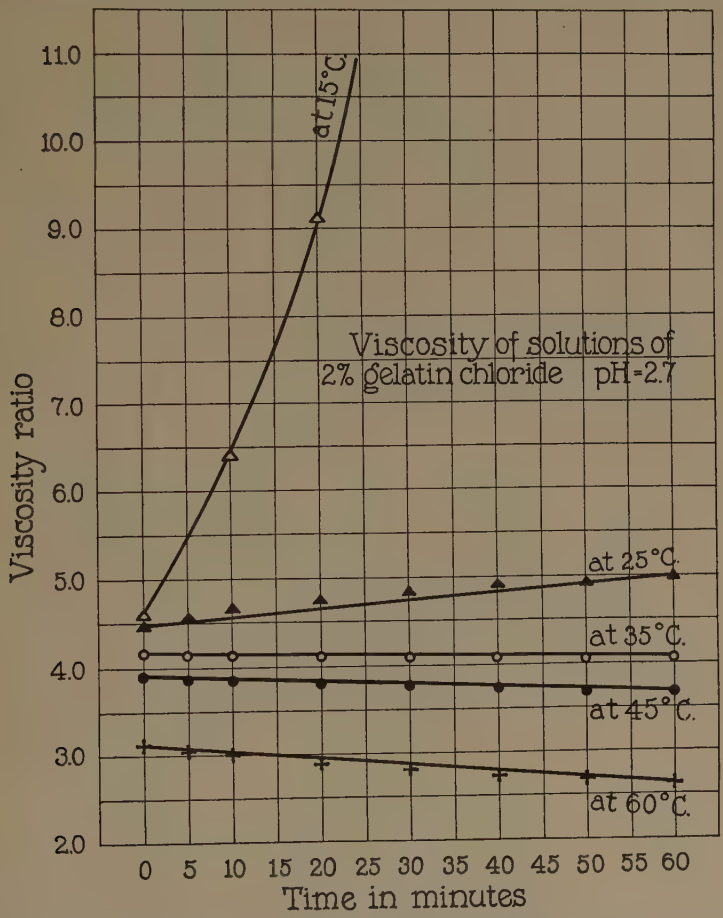

FIG. 57.—Influence of temperature on the variation of viscosity of gelatin solutions on standing. Below 35°C. the viscosity of a 2 per cent gelatin chloride solution of pH 2.7 no longer increases but diminishes on standing.

15° the viscosity increased rapidly on standing; at 25° it increased on standing but less rapidly; at 35° or above it diminished on standing, the more rapidly the higher the temperature. The

temperature at which the two opposite processes—the formation and the melting of micellæ—occur equally rapidly in a 2 per cent solution of gelatin chloride of pH 2.7 lies between 25 and 35°C.

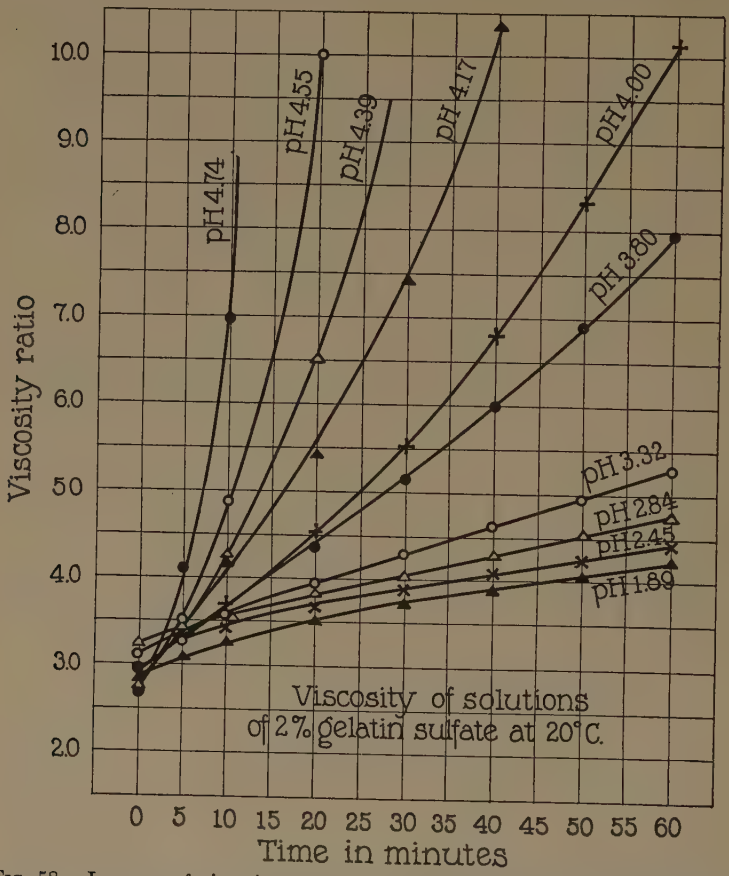

FIG. 58.—Increase of viscosity of gelatin sulphate solution of different pH on standing. The increase is most rapid at the isoelectric point, thus proving that the acid retards or prevents the formation of submicroscopic solid particles of jelly on standing.

When acid is added to powdered isoelectric gelatin the time required to dissolve the particles diminishes at a given temperature with increasing hydrogen ion concentration of the solution and this tendency of the particles to dissolve with increasing hydrogen

ion concentration shows no maximum as does the swelling. Hence we should expect that the more acid is added to a 0.5 per cent

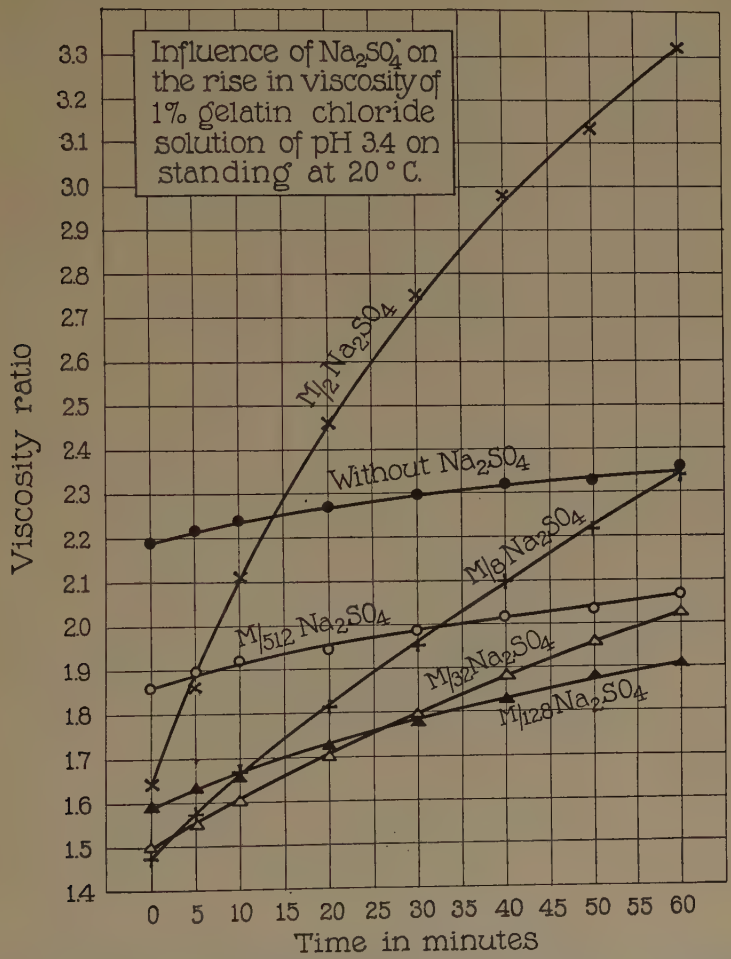
Influence of Na_2SO_4 on the rise in viscosity of 1% gelatin chloride solution of pH 3.4 on standing at 20° C.
Viscosity ratio
Time in minutes
M/2 Na_2SO_4
Without Na_2SO_4
M/8 Na_2SO_4
M/512 Na_2SO_4
M/32 Na_2SO_4
M/128 Na_2SO_4

FIG. 59.—Showing that concentrations of Na_2SO_4 of M/32 and above cause an increase in the viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

solution of isoelectric gelatin the less the viscosity will increase on standing at a given temperature, *e.g.*, 20°C., since the more acid is added to isoelectric gelatin the greater the tendency of

the solid jelly particles already existing to dissolve; while the tendency of the isolated gelatin molecules or ions to adhere to each other is not increased. It should follow that on standing the viscosity of a 0.5 per cent solution of gelatin chloride or gelatin sulphate will increase the less at 20° the lower the pH of the solution. Figure 58 shows that this is the case.

In Chap. XIV we shall see that the rate of solution of powdered gelatin in water is influenced in a different way by different salts. Na_2SO_4 diminishes the rate of solution of powdered gelatin chloride when the concentration of Na_2SO_4 exceeds M/64; and the diminution is the greater the higher the concentration; while $CaCl_2$ accelerates the rate of solution of powdered gelatin chloride when the concentration of $CaCl_2$ exceeds M/4.

Gelatin chloride solutions of pH 3.4, containing 1 gm. of originally isoelectric gelatin in 100 c.c. solution, were made up in various concentrations of Na_2SO_4 and $CaCl_2$. The solutions were rapidly heated to 45° and rapidly cooled to 20°C. and kept at this temperature for 1 hour. The time of outflow of the solution through a viscometer was measured immediately and in intervals of 5 or 10 minutes. The time of outflow of water through the viscometer at 20° was 61 seconds.

The viscosity of a gelatin chloride solution of pH 3.4 rises gradually but very slowly (uppermost curve in Fig. 59) and the rate of increase of viscosity on standing is not materially altered in M/512 Na_2SO_4 and only little in M/128 Na_2SO_4. In M/32 Na_2SO_4 the viscosity increases more rapidly on standing, in M/8 Na_2SO_4 still more rapidly, and in M/2 Na_2SO_4 very sharply. This is exactly what we should expect, since the Na_2SO_4 causes a diminution of the rate of solution of gelatin chloride as soon as the concentration of Na_2SO_4 is above M/64. In such solutions the rate of solution of micellæ will be less and less, and since new micellæ are constantly formed at 20°C. the viscosity will rise more rapidly on standing when the solution contains Na_2SO_4 in concentrations above M/64 than when the solution contains less Na_2SO_4 or none at all.

Figure 60 shows that $CaCl_2$ in concentrations up to M/8 does not alter the increase in viscosity of gelatin chloride solution on standing, but that the viscosity of gelatin chloride of pH 3.4 no

longer increases on standing when the concentration of $CaCl_2$ is M/2 or 1 M. In this concentration $CaCl_2$ causes a slight increase in the rate of solution of gelatin chloride.

NaCl causes no change in the rate of solution of gelatin chloride as long as the concentration of NaCl does not exceed 1 M. Above this concentration it causes coagulation and the viscosity

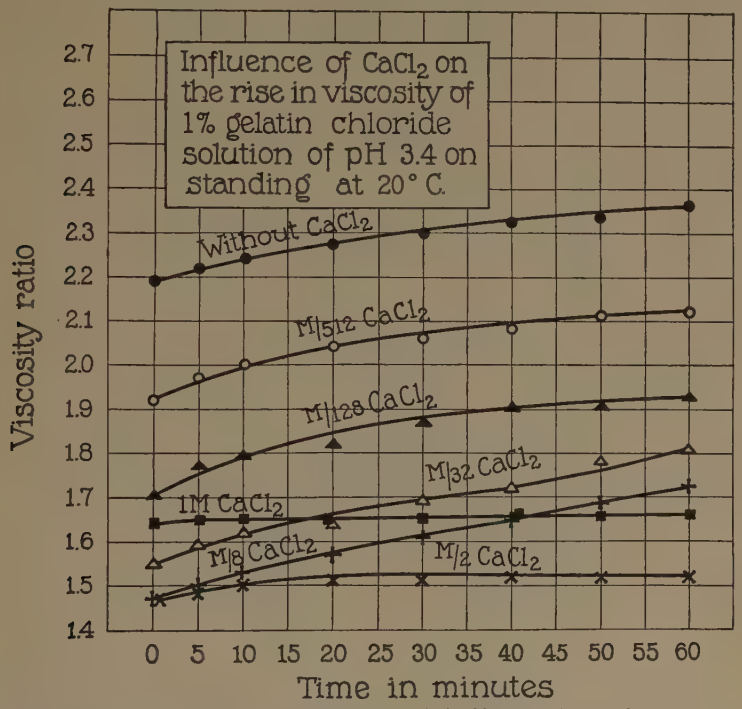

FIG. 60.—Showing that concentrations of $CaCl_2$ or M/2 or above prevent the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

can no longer be measured. Hence NaCl in concentrations up to 1 M should not alter the rate of increase of viscosity of gelatin chloride solutions on standing. Figure 61 shows that this is correct.

The simplest method of melting solid particles of jelly is by heating to 45°C. If, therefore, the striking increase in viscosity which occurs when a 0.5 per cent solution of isoelectric gelatin is

kept standing for a day at a temperature of, *e.g.*, 10°C., is due to the formation of particles of solid jelly, then if this solution is heated to 45°C. and cooled rapidly to 20°C. the majority of these solid particles should have melted and dissolved into isolated

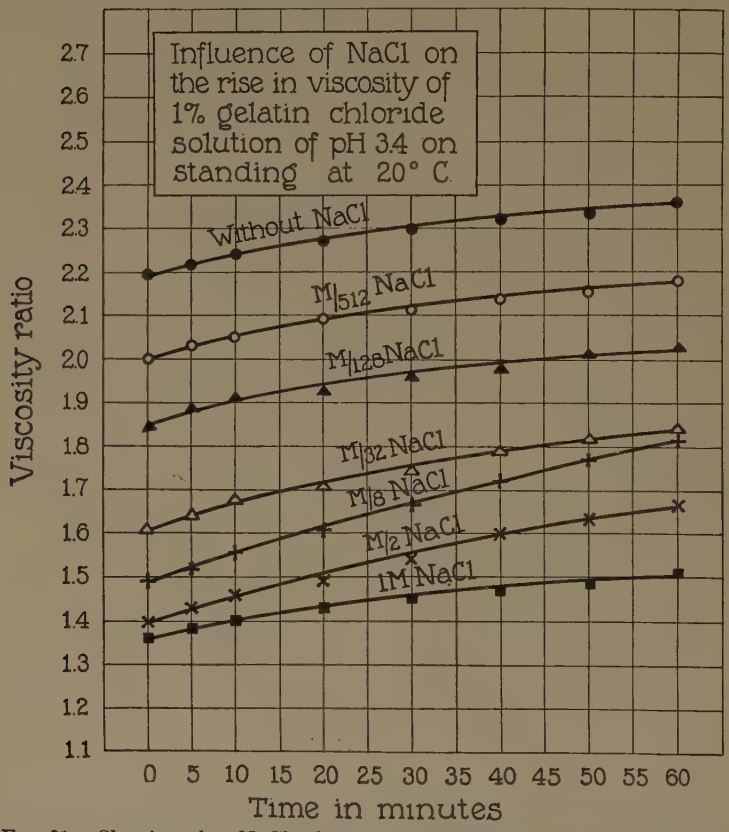

FIG. 61.—Showing that NaCl solutions up to a concentration of 1M have no effect on the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

ions or molecules. Hence such a solution when cooled rapidly to 20° should show at this temperature a considerably lower viscosity than the same solution shows at 20° when it is brought to this temperature directly from 10°C. without previous heating to 45°C. The experiment represented in Fig. 62 shows that this is the case.

These experiments then support the conclusion that the high viscosity of gelatin solutions and the influence of electrolytes on this viscosity is due to the fact that these solutions contain submicroscopic particles of solid jelly (micellæ) capable of occluding large amounts of water the quantity of which is regulated by the Donnan equilibrium.

4. The pH influences the viscosity of casein chloride solutions in a similar way to that in which it influences gelatin chloride

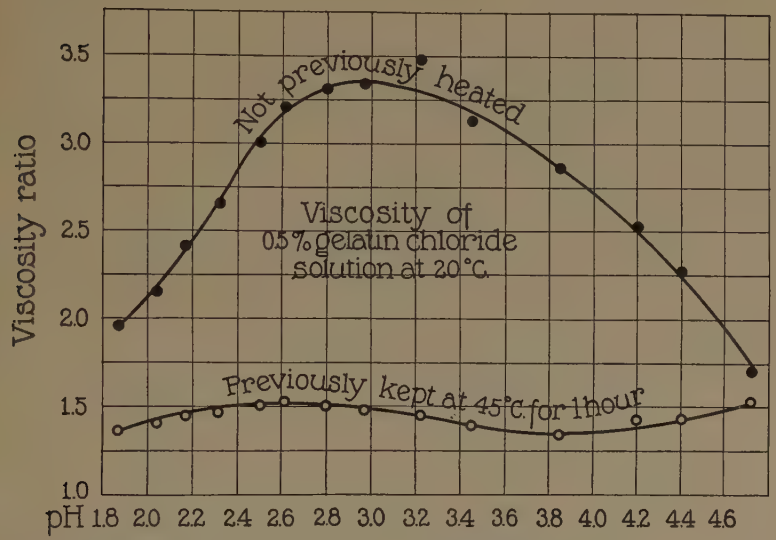

FIG. 62.—Showing that previous heating diminishes the viscosity of 0.5 per cent solutions of gelatin chloride.

solutions; and the depressing effect of neutral salts on the viscosity of casein chloride solutions is similar to that of the addition of salts on the osmotic pressure of gelatin chloride. Casein chloride solutions have no tendency to set to a jelly, but they have one feature in common with gelatin solutions, namely, the existence of particles capable of occluding water, the amount of which is regulated by the Donnan equilibrium. As a consequence, casein chloride solutions have a comparatively high viscosity which is influenced by electrolytes in the way characteristic for the Donnan equilibrium. The existence of such

particles in the casein chloride solution is indicated by the opacity of the solution.

The material used in our experiments was a fine dry powder of nearly isoelectric casein prepared after Van Slyke and Baker. Particles of equal size of grain (between mesh 100 and 120) were sifted out and 1 gm. of such powder was put into 100 c.c. each of solutions of HCl of different concentration to bring the casein to varying pH. A microscopic examination of the granules

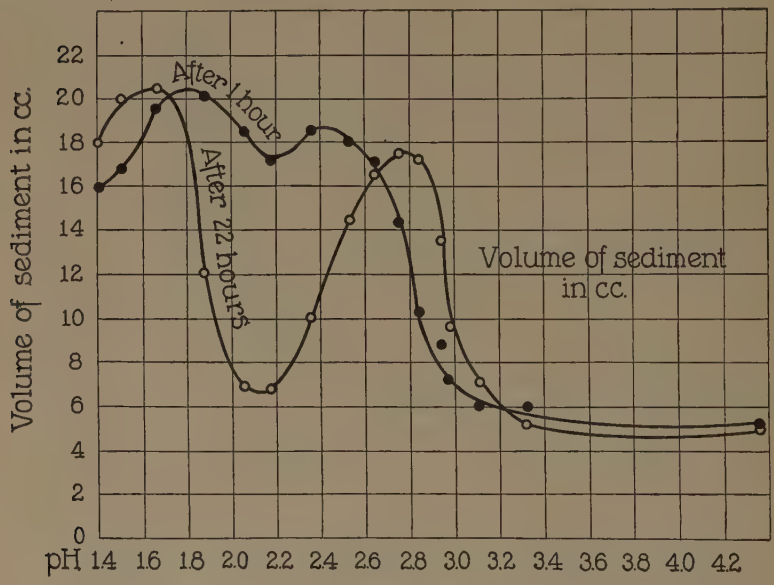

FIG. 63.—Swelling and solution of casein chloride in 1 and 22 hours at 20°C.

showed that they underwent a swelling which was a minimum at the isoelectric point, which increased with increasing hydrogen ion concentration until it reached a maximum, and which then diminished again with a further increase in the hydrogen ion concentration (see Chap. XV). Hence, the volume of the casein particles suspended in the HCl varied in a similar way with the pH as the volume of suspended particles of gelatin.

This swelling could also be observed when the suspension was put into 100 c.c. graduates and the suspended particles were allowed to settle. The volume of the sediment was a minimum at the isoelectric point increasing with increasing hydrogen ion

concentration of the solution and finally decreasing again. But the curves of swelling and of volume of sediment were only parallel at the beginning of the experiment, since the swelling (which occurred at once) was followed by some of the casein going into solution or into suspension. The longer the experiment lasted the smaller the volume of the sediment became and the larger the mass which went into the supernatant solution. This is expressed in Fig. 63. The upper curve represents the volume of the sediment after 1 hour. The suspension of 1 gm. of

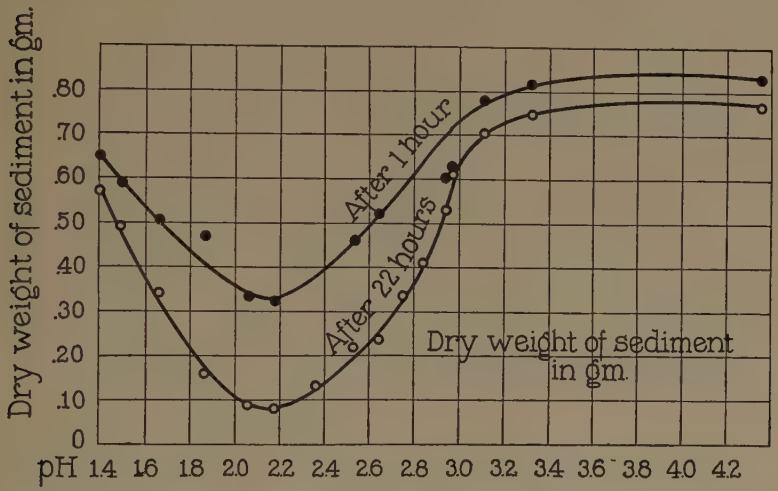

FIG. 64.—Dry weight of sediment of casein chloride solutions after 1 and 22 hours.

casein in 100 c.c. of HCl of different concentration had been kept for 1 hour at 20°, had been shaken repeatedly but not frequently, and the suspension was then passed into 100 c.c. graduates and allowed to settle at 20°C. After 2 hours the volume of the sediment was measured and the volumes are the ordinates of the curve marked "after 1 hour" in Fig. 63. A similar experiment was made in which the suspension of casein was kept for 22 hours at 20°C. and was allowed to settle during 6 hours also at 20°C. The volumes are the ordinates of the second curve in Fig. 63, marked "after 22 hours." The abscissæ are the pH of the total solution and suspension.

The curve "after 1 hour" is clear, since it is chiefly the expres-

sion of the variation of the degree of swelling of the casein particles, not as much having gone into solution as after 22 hours. We notice that the volume occupied by the solid particles in the 1-hour curve is a minimum at the isoelectric point, that it rises steeply after pH 3.1, that it drops at 2.2, and that a second drop commences at pH 1.8. The two drops have a different cause. The drop at pH 1.8 is due to a diminution of the degree of swelling of the sediment, while the drop at 2.2 in the 1-hour curve is due to the fact that at pH 2.2, where the solubility of casein chloride is a maximum, some of the casein chloride has gone into solution. This conclusion is supported by the fact that the drop at 2.2 increases in time and is very considerable after 22 hours (see Fig. 63), while otherwise the 1-hour and the 22-hour curves show only minor differences.

The proof that this interpretation in the volume curves of Fig. 63 is correct is furnished by Fig. 64, where the ordinates are the dry weights of the sediments, the volumes of which are given in Fig. 63. One gram of powdered casein had when dried for 24 hours at between 90 and 100°C. a dry weight of 0.87 gm.

That part of the casein chloride which goes into the supernatant liquid (*i.e.*, which is not contained in the sediment) consists of two constituents, namely, first, solid submicroscopic particles in suspension which in due time would have settled, and second, isolated casein ions and molecules. The solid particles in the supernatant liquid (unless they are below the limit required to occlude water) undergo the same swelling under the influence of the Donnan equilibrium as the particles of the sediment. In addition, however, we have individual casein ions in solution (the molecules being probably insoluble since isoelectric casein is practically insoluble) but these ions cannot undergo any swelling and hence do not add materially to the volume and the viscosity. As a consequence, the more solid particles of casein chloride are dissolved into isolated casein ions or particles too small to occlude water, the more the relative volume occupied by the casein in the solution should be diminished, and this should be accompanied by a diminution in viscosity. If our theory of the origin of the viscosity of the gelatin solutions is correct, it should be possible to prove that where the solubility of the casein chloride solution is a maximum the viscosity curve shows a drop.

The correctness of this inference is supported by the viscosity curves in Fig. 65, which represent the viscosity after 1 hour and after 22 hours. The experiments are the same as those referred to in Figs. 63 and 64. The viscosity of the total suspension and solution was measured in a straight viscosimeter with a time of outflow for water of 48.4 seconds at 20°C. The curve for the viscosities after 1 hour is the expression chiefly of the swelling, since casein chloride goes only slowly into solution at 20°C. The curve is almost continuous and has its maximum in the region between pH 2.1 and 2.4, where also the swelling is a

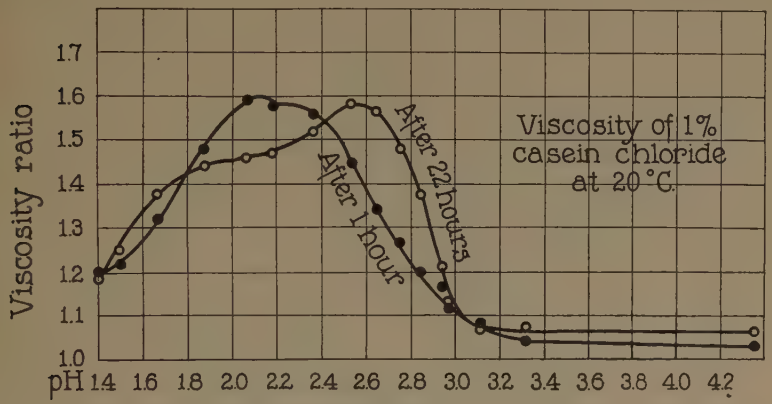

FIG. 65.—Viscosity of 1 per cent casein chloride solutions after 1 and 22 hours at 20°C.

maximum. There is, however, a slight depression at pH 2.2, where the solubility of the casein is a maximum.

The curve for the viscosities, Fig. 65, after 22 hours shows, however, a distinct saddle at pH 2.2 where the solubility of casein chloride is a maximum. This agrees with the assumption that the high viscosity is due to swollen particles of casein, a certain quantity of which had been dissolved at or near pH 2.2. This solution of the particles capable of swelling beneath that size where they no longer can occlude water must diminish the relative volume of the casein and cause a diminution of the viscosity. Below a pH of 1.8, where the solubility of the casein is considerably diminished, the 1-hour and the 22-hours viscosity curves (Fig. 65) no longer differ materially. As a consequence of the

saddle the maximum of the viscosity curve after 22 hours now lies at pH 2.6.

Since the point at issue, namely, the diminution of the viscosity when solid submicroscopic particles, capable of swelling, are dissolved into particles so small that they no longer can occlude water, is so fundamental for the theories of viscosity and of colloidal behavior in general, it seemed necessary to look for a more striking proof than that given in the experiment quoted. For this purpose measurements were made on 1 per cent casein chloride solutions prepared from very finely powdered casein particles sifted through a 200-mesh sieve. In order to get a

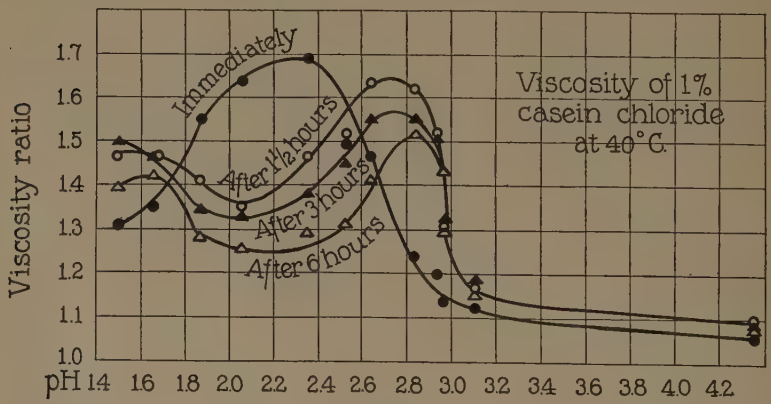

FIG. 66.—Diminution of viscosity through solution of solid particles of casein chloride.

more rapid solution of the particles the experiment was carried out at 40°C. The time of outflow of water through the viscometer at 40° was 35.5 seconds. Figure 66 gives the results. The viscosity measurements were made at four different times, namely: first, immediately after the powdered casein was put into the HCl; then after 1½, 3, and 6 hours. During this time the casein chloride solutions were kept at 40°C. The viscosity curve taken immediately after the suspensions were prepared is continuous and is the expression of the swelling which occurred in the few minutes which elapsed in the preparation of the suspensions and during which the casein was at 40°C. The maximum swelling occurred at about pH 2.3. At this time the amount of

casein dissolved into separate casein ions was negligible. The curve resembles the 1-hour curve in Fig. 65. After 1½ hours the second measurements of viscosity were taken, and the reader will notice from Fig. 66 that the viscosity had dropped considerably in the neighborhood of pH 2.2 where the solubility of casein chloride is the greatest, and the maximum depression is at pH 2.1 where also the solubility is a maximum. With a further lowering of the pH the viscosity rises again. The maximal viscosity in the 1½-hours series is now at pH of about 2.7 or 2.8 where it

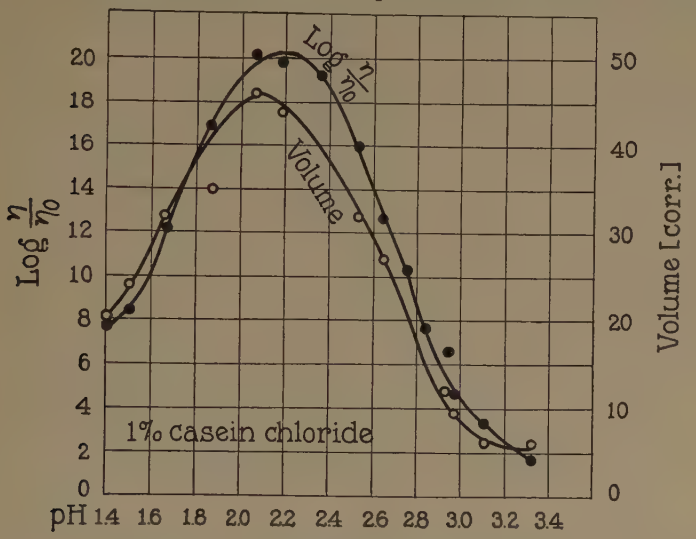

FIG. 67.—Similarity of curves for log $\frac{\eta}{\eta_0}$ and for relative volume of casein chloride in solutions.

was also in the 22-hours series in Fig. 65. The later viscosity measurements, after 3 and 6 hours (Fig. 66) confirm these conclusions.

5. It is of interest to see whether or not Arrhenius's formula can account for the influence of electrolytes on the viscosity of casein suspensions. If this were the case, the curves representing log $\frac{\eta}{\eta_0}$ should run parallel to curves representing the relative volume occupied by the casein in the solution. We get the values of log $\frac{\eta}{\eta_0}$ from our observations of the relative viscosity

which give us $\frac{\eta}{\eta_0}$, and we can calculate the volume from the measured volume of the sediment plus the calculated volume of the casein in the supernatant liquid. The latter value is obtained by deducting the dry weight of the sediment from the (known) dry weight of the whole mass of casein put into the water (1 gm. powdered casein, dry weight = 0.87 gm.); assuming that the casein in the supernatant liquid consists exclusively of suspended particles. This is partly correct for a 1-hour experiment at 20°. The ordinates in Fig. 67 represent the values for volume thus corrected and the values for log $\frac{\eta}{\eta_0}$ while the abscissæ are the pH of the suspensions. The two curves are almost parallel.

It should be stated that these corrected volumes of casein include a certain amount of water between the granules. We are, however, in this case not concerned with the absolute but only the relative volume occupied by the casein.

When NaCl is added in different concentrations to a casein chloride solution it is noticed that the viscosity is diminished as it is in the case of solutions of gelatin chloride. We shall see in Chap. XV that this diminution of viscosity is accompanied by a diminution in the degree of swelling of the individual particles of casein which is parallel to the depression of the viscosity.

One gram of powdered casein was put into 100 c.c. of H_2O containing 12.5 c.c. of 0.1 N HCl, and NaCl in concentrations varying from 0 to M/4. The mixture was shaken occasionally and kept for 16 hours at 20°. Then the viscosity, volume of sediment (after settling for 24 hours), dry weight of sediment (after deduction of the free NaCl contained in the sediment) were determined. When the volume and the values for log $\frac{\eta}{\eta_0}$ are plotted as ordinates over the concentrations as abscissæ, it is found that the two curves agree fairly well (Fig. 68) except where no or little salt was added and where therefore some casein particles had been completely dissolved. In this solution the calculated volume was too high and our curves express the fact. From these experiments we may conclude that the influence of electrolytes on the viscosity of casein solutions or suspensions is due to the swelling of particles of casein suspended in the solu-

tion of casein and that the volume of these particles is regulated by the Donnan equilibrium.

6. These experiments leave little doubt that the high viscosity of certain protein solutions, such as gelatin or casein, is due to the existence of solid particles occluding large quantities of water, the amount of which is regulated by the Donnan equilibrium, while the isolated ions of proteins in solution or the particles too small to occlude water have no share in the causation of high viscosities.

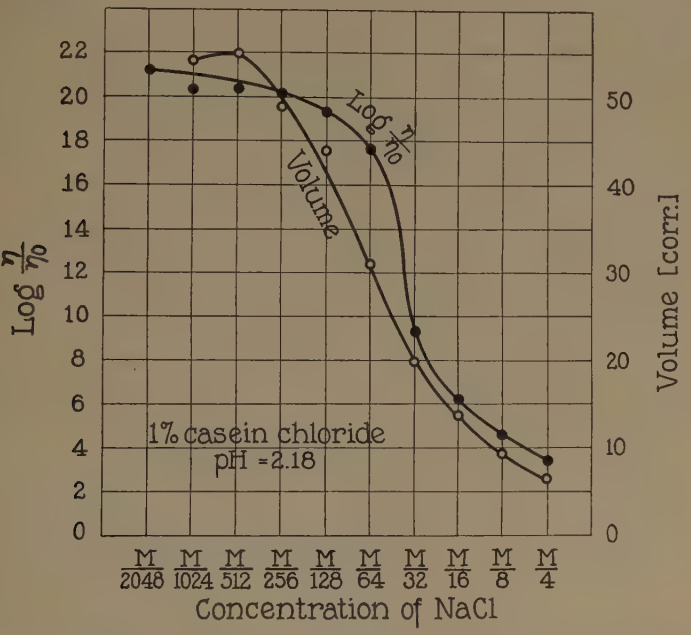
Log η/η0
Volume [corr.]
Log η/η0
Volume
1% casein chloride
pH =2.18
Concentration of NaCl
M/2048 M/1024 M/512 M/256 M/128 M/64 M/32 M/16 M/8 M/4
0 2 4 6 8 10 12 14 16 18 20 22
0 10 20 30 40 50

FIG. 68.—Similarity of curves for log $\frac{\eta}{\eta_0}$ and for relative volume of casein chloride in solutions.

The quantities of water which can be occluded in a solid jelly of gelatin are enormous. If we assume the molecular weight of gelatin to be of the order of magnitude of about 12,000, a solid gel of 1 per cent originally isoelectric gelatin contains over 60,000 molecules of water to 1 molecule of gelatin. It is out of the question that such masses of water could be held by the secondary valency forces of the gelatin and water molecules. Casein particles occlude much less water and for this reason the

viscosity of casein chloride solutions never becomes as high as that of gelatin solutions containing equal masses of protein per 100 c.c. of solution.

All the experiments described agree with the occlusion theory but not with the hydration theory. Thus the fact that the viscosity of a 0.5 per cent solution of isoelectric gelatin increases rapidly at a temperature of 20°C. or below cannot possibly be explained on the basis of the hydration theory since isoelectric gelatin is not ionized. It might be explained on the basis of another suggestion which attributes to the gelatin solution a similar structure to that possessed by the solid jelly of gelatin. This idea would lead us to the assumption that in addition to the source of viscosity due to the relative volume of the protein solution there exists a second type peculiar to protein solutions which has no connection with the volume.

"Bearing in mind the possibility that protein solutions may contain a preformed molecular structure analogous to that of the jellies or coagula which they can form, we are strongly impelled towards the belief that the type of viscosity which solutions of proteins exhibit may in some manner owe its existence to this structure, and not to the type of internal friction which hinders molecular and ionic motion. Thus a netlike structure, such as a tennis net, will offer no hindrance to the passage through it of a quickly moving body which is smaller than its meshes, other than that which is due to the fact that the material which composes the net occupies a small fraction of the area which the body must traverse, but to any force which involves deformation of the structure, for instance, a force which seeks to drag it through a small tube, it will offer a very considerable resistance."[1]

This theory becomes untenable in the case of suspensions of powdered gelatin and of casein chloride which have no tendency to set to a jelly. It fails, moreover, to account for the fact that the influence of pH on the viscosity resembles that on the osmotic pressure of gelatin solutions. The assumption of a second type of viscosity independent of the relative volume occupied by the solute becomes unnecessary, since the theories of Einstein and of Arrhenius respectively, which derive the viscosity from the relative volume, suffice to account for all the phenomena observed.

[1] Robertson, T. B., "The Physical Chemistry of Proteins," pp. 324–25, New York, London, Bombay, Calcutta, and Madras, 1918.

We therefore arrive at the conclusion that where the hydrogen ion concentration, the valency of ions, and the concentration of salts influence the viscosity of protein solutions in a similar way to that in which they influence the osmotic pressure, this influence on viscosity is in reality an influence of electrolytes on the swelling of solid submicroscopic protein particles contained in the solution.

CHAPTER XIII

A RECIPROCAL RELATION BETWEEN THE OSMOTIC PRESSURE AND THE VISCOSITY OF GELATIN SOLUTIONS[1]

1. The experiments in the preceding chapter have led to the conclusion that proteins form true solutions consisting of isolated protein ions and molecules which may or may not contain in addition to the isolated ions and molecules submicroscopic particles capable of occluding water and giving rise to a Donnan equilibrium. Only when a protein solution contains particles of this latter type do we notice a comparatively high viscosity and a similar influence of electrolytes on viscosity as on osmotic pressure. Solutions of crystalline egg albumin of not too high a concentration have a comparatively low viscosity which is not affected in the typical way by electrolytes and this leads to the conclusion that these solutions consist chiefly of isolated ions and molecules or of particles too small to occlude water. If this conclusion is justified, we are forced to the further conclusion that the influence of electrolytes on the osmotic pressure of protein solutions is determined by the isolated ions of a protein solution and not by the submicroscopic particles capable of occluding water, *i.e.*, the micellæ, since solutions of crystalline egg albumin show the influence of electrolytes on their osmotic pressure in a striking way. It would further follow that in case of a gelatin solution where both isolated ions and submicroscopic micellæ are supposed to exist the isolated ions are responsible for the influence of electrolytes on the osmotic pressure of the solution while the submicroscopic particles of solid jelly capable of occluding water are responsible for the influence of electrolytes on the viscosity of gelatin solutions. In other words, wherever there exists a reversible aggregate formation from isolated protein ions in solution

[1] Loeb, J., *J. Gen. Physiol.*, vol. 4, p. 97, 1921–22.

there should exist a reciprocal relation between the viscosity and the osmotic pressure of the solution since, the transformation of the submicroscopic particles of solid jelly should lower the viscosity and raise the osmotic pressure of a gelatin solution and *vice versa*. It can be shown that this conclusion is supported by observations on gelatin solutions.

It was noticed in the preceding chapter that the viscosity of solutions of gelatin chloride does not always increase on standing but that it diminishes when the temperature exceeds a certain limit. This was shown for a 2 per cent solution of gelatin chloride of pH 2.7 in Fig. 57. The viscosity of such a solution increases very rapidly on standing at 15°C., much less rapidly at 25°C., but diminishes when kept at a temperature above 35°C., and the more rapidly the higher the temperature. This we assume to be due to the fact that at a temperature above 35°C. the rate of melting of submicroscopic particles of solid jelly exceeds the rate of their formation from isolated ions or molecules.

Several liters of a 0.55 per cent solution of isoelectric gelatin were kept at about 10°C. for 48 hours and at 20°C. for the next 24 hours. Then the stock solution was divided into two parts. The one part was subdivided into doses of 90 c.c. each, and each was brought to a different pH by adding 10 c.c. containing different quantities of HCl. In this way the concentration of originally isoelectric gelatin was, therefore, in every case 0.5 per cent. The second portion was treated in the same way except that before adding the acid the gelatin was kept for 1 hour at 45°C. This was done to melt part of the submicroscopic pieces of jelly assumed to exist in the solution, and thus to increase the concentration of the isolated ions and molecules and to diminish the relative quantity of solid submicroscopic particles responsible for the high viscosity characteristic of gelatin solutions. After this second portion of the stock solution of isoelectric gelatin had been kept for 1 hour at 45°C. it was rapidly cooled to 20°C., the HCl was added in the way described for the first portion and the solutions were put into collodion bags to measure the osmotic pressure. Each collodion bag contained about 50 c.c. of gelatin solution. The temperature now remained constant at 20°C. for both sets of experiments. It was noticeable from the first that the osmotic pressure of the gelatin solution

which had been kept for 1 hour at 45° and which was therefore supposed to have melted into smaller particles was higher than that of the gelatin solution not previously heated. Figure 69 shows the result after 22 hours. The maximum osmotic pressure was for the gelatin solution that had been previously heated 200 mm. H_2O, while it was only 170 mm. for the other gelatin solution not previously heated to 45°C.

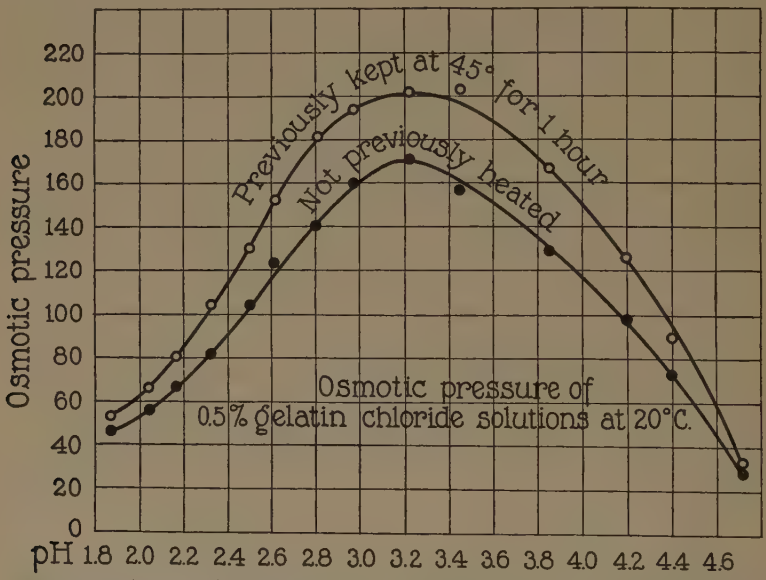

FIG. 69.—Showing that the osmotic pressure of a solution of gelatin chloride which has been previously heated to 45°C. for 1 hour and then rapidly cooled to 20°C. is higher than the osmotic pressure of the same solution of gelatin chloride not previously heated.

Then the viscosities were determined at 20° and they gave the opposite result (Fig. 62 of the preceding chapter), the viscosities being considerably higher in the solutions not previously heated to 45° than in the solutions previously heated. This experiment then confirms our expectation that there exists a reciprocal relation between the viscosity of protein solutions and their osmotic pressure, inasmuch as a transformation of solid submicroscopic particles of jelly into isolated protein ions and molecules diminishes the viscosity but increases the osmotic pressure.

As far as the quantitative relations are concerned, the differ-

ence in viscosity (Fig. 62) is more striking than the difference in osmotic pressure (Fig. 69). This is possibly connected with the fact that the lowering in viscosity due to heating to 45°C. was measured immediately after the temperature had reached 20°C. again, while the osmotic pressure of the same solutions was measured after the solutions had been standing for 22 hours at 20°C. During this time a considerable formation of submicroscopic particles of solid jelly had probably occurred in the solutions previously heated to 45°C.

It was expected that when we put a collodion bag filled with a 1 per cent solution of gelatin of *e.g.*, pH 3.5, which had been kept for 1 hour at 45° and cooled to 20° into a beaker containing a 1 per cent solution of the identical gelatin chloride solution of pH 3.5, but which had not been heated to 45°C. before being brought to 20°C., water would diffuse from the latter into the former solution. This experiment was carried out with a positive result.

These experiments support the idea expressed in the preceding chapter that protein solutions are true solutions which may or may not contain solid particles of protein capable of swelling. In the case of gelatin solutions the formation of submicroscopic particles of solid jelly from isolated molecules or ions is a reversible process, and this leads in this case to a reciprocal variation of osmotic pressure and viscosity of such solutions.

This probably explains a phenomenon which has puzzled the writer for a long time, namely that the osmotic pressures of gelatin solutions of the same pH and concentration of originally isoelectric gelatin showed occasionally variations for which he could not account. It now becomes probable that this was due to a factor which was not taken into consideration, namely, that on standing at room temperature a gradual transformation of isolated molecules or ions into larger aggregates takes place, which must diminish the osmotic pressure but increase the viscosity. This source of variation was eliminated in the viscosity experiments in which the gelatin solution was always heated first to 45°C. and then as soon as this temperature was reached the solution was cooled to the temperature desired for the viscosity measurements. It is probable that the same uniformity of treatment is also required for the osmotic pressure experiments.

This reciprocal relation between osmotic pressure and viscosity

exists probably also in the case of solutions of casein salts. Solutions of Na caseinate are less opaque than those of casein chloride (of the same concentration of originally isoelectric casein) which indicates that the Na caseinate solution contains more isolated casein ions and fewer submicroscopic solid particles than the solution of casein chloride.

The writer had already shown in a preceding chapter that the maximal viscosity of a 1 per cent solution of casein chloride is higher than the viscosity of solutions of Na caseinate of equal concentration of originally isoelectric casein, while the osmotic pressures of solutions of the two salts show exactly the reverse relation, the maximal osmotic pressure of a 1 per cent solution of Na caseinate being almost 700 mm. H_2O while the maximal osmotic pressure of a 1 per cent solution of casein chloride is only about 200 mm.

The solutions of crystalline egg albumin seem to consist (at ordinary temperature and at not too high a concentration of albumin and of the hydrogen ions) exclusively or almost exclusively of isolated molecules or ions. Since the latter cannot diffuse through a collodion membrane they give rise to a Donnan equilibrium across the membrane and hence only the osmotic pressure of solutions of salts of crystalline egg albumin is influenced by electrolytes in the way demanded, while the viscosity shows such an influence only to a negligible degree.

2. It should be possible to give a more striking confirmation of the reciprocal relation between the viscosity and the osmotic pressure if we replace in a gelatin solution part of the dissolved gelatin by equal weight of powdered gelatin. Such a substitution should increase the viscosity and diminish the osmotic pressure of the solution.

Figure 70 shows that the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin diminishes the more the more we replace the dissolved gelatin by small granules of powdered gelatin. The ordinates of the upper curve represent the values of the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin at different pH, the pH serving as abscissæ of the curves. The acid used was HCl, and the curve is the usual one. At the beginning of the experiment the gelatin solution was rapidly heated to a temperature of 45°C. and rapidly cooled to

20°C. and then kept at that temperature throughout the entire experiment. The pH is that of the gelatin solution at the end of the experiment.

The middle curve represents an experiment in which 0.5 gm. of the isoelectric gelatin in solution was replaced by 0.5 gm. of

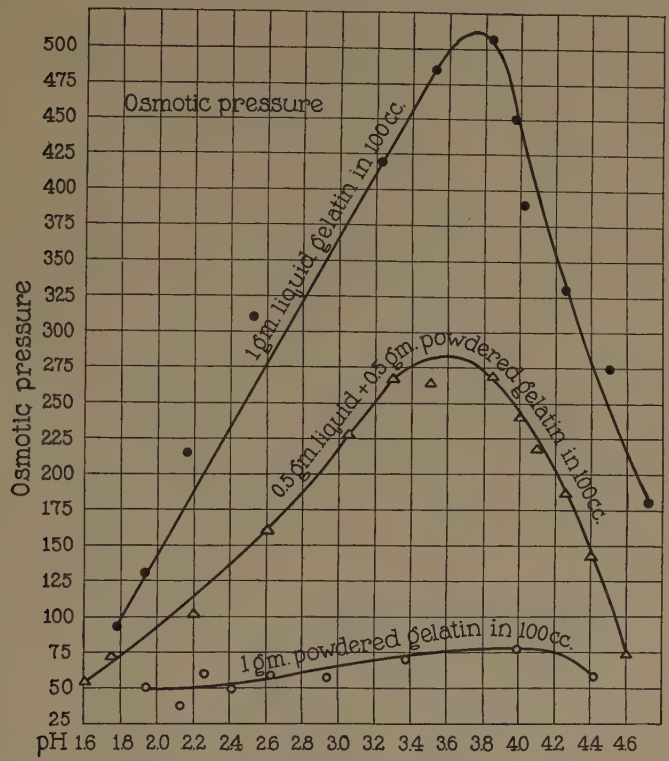

FIG. 70.—A suspension of 1 gm. of a fine powder of gelatin in 100 c.c. of water has practically no osmotic pressure (lowest curve), while a solution of 1 gm. of the same gelatin has a maximal osmotic pressure of over 500 mm. (uppermost curve). A mixture of 0.5 gm. of powdered and 0.5 gm. of liquid gelatin in 100 c.c. water has practically the osmotic pressure of the 0.5 per cent liquid gelatin in 100 c.c. of water (middle curve).

isoelectric powdered gelatin. The latter did not contribute to the osmotic pressure, the observed osmotic pressure being due to the isolated ions of the 0.5 per cent gelatin in solution which determined the Donnan effect, and in addition to the gas pres-

sure of the isolated gelatin ions and the isolated gelatin molecules. Theoretically, of course, the coarse particles of gelatin also participate in the osmotic pressure but this effect is negligible on account of the small number of such particles. (The gelatin particles used were of grain size slightly above $\frac{1}{60}$ of an inch diameter.) At the beginning of the experiment the 0.5 per cent solution of gelatin was rapidly heated to 45°C. and rapidly cooled to 20°C., and then the powdered gelatin was added. The pH is that of the 0.5 per cent gelatin solution at the end of the experiment.

The lowest curve represents the osmotic pressure of 1 gm. of powdered isoelectric particles in 100 c.c. of HCl of different pH. The slight osmotic pressure observed is probably due to the fact that a little of the gelatin went gradually into solution. This apparently happened to a less extent in a repetition of this experiment and the osmotic pressures observed were still lower than in the lowest curve in Fig. 70. All these osmotic pressure experiments were made in a thermostat at 20°C.

The viscosity is affected in exactly the opposite sense from the osmotic pressure if part of the dissolved gelatin is replaced by solid particles of gelatin. The more dissolved gelatin is replaced by solid particles of gelatin the higher the viscosity, a result to be expected from the experiments and conclusions already stated.

Solutions of 0.5, 0.625, 0.750, 0.875, and 1.0 gm. of isoelectric gelatin were heated quickly to 45°C. and cooled quickly to 20°C., and so much powdered gelatin of pH 7.0 was added as to bring the total gelatin in 100 c.c. to 1 gm.; *i.e.*, to a 0.5 per cent solution of gelatin was added 0.5 gm. of powdered gelatin (between mesh sizes 100 and 120), and to a 0.875 per cent solution of liquid gelatin was added 0.125 gm. of powdered gelatin, while no powdered gelatin was added to the 1 per cent solution of liquid gelatin. The different mixtures were brought to different pH through the addition of different quantities of HCl and the solutions were allowed to stand for 1 hour before the viscosities were measured in order to give the powdered gelatin a chance to swell. The results of the measurements are represented in Fig. 71. The reader will see that within the range of the pH between 3.6 and 1.4 the viscosity is the greater, the more liquid gelatin is

replaced by powdered gelatin. This supports the idea that the influence of electrolytes on the viscosity of gelatin solutions is due to the influence of the electrolytes on the swelling of solid submicroscopic particles of gel in the solution.

The nature of the curves in Fig. 71 between pH 4.6 and 3.8 requires an explanation. The curves are here the lower the

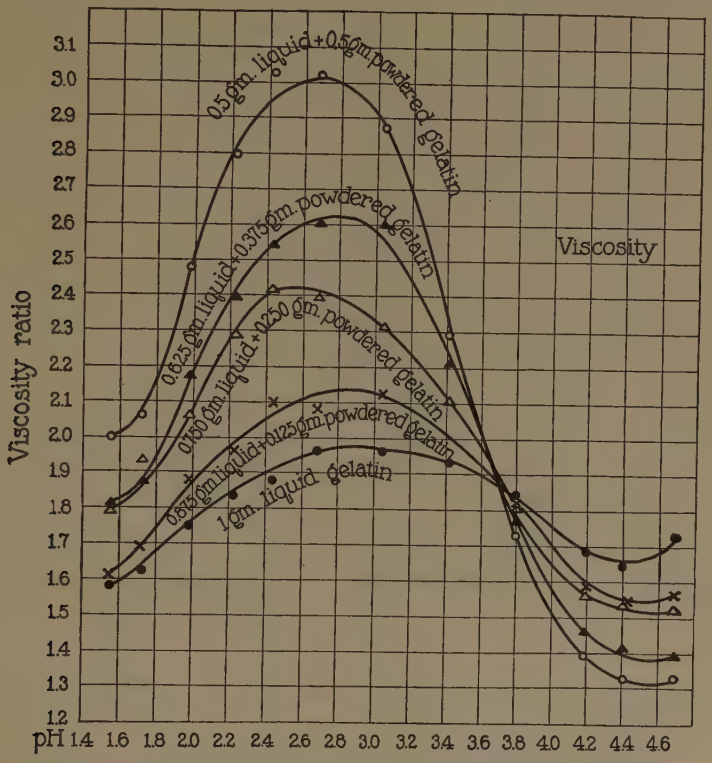
Viscosity ratio
pH
Viscosity
0.5 gm. liquid + 0.5 gm. powdered gelatin
0.625 gm. liquid + 0.375 gm. powdered gelatin
0.750 gm. liquid + 0.250 gm. powdered gelatin
0.875 gm. liquid + 0.125 gm. powdered gelatin
1 gm. liquid gelatin

FIG. 71.—The influence of replacing liquid by powdered gelatin on viscosity is exactly the reverse as on osmotic pressure. The more the powdered particles replace the liquid gelatin the higher the viscosity.

more liquid gelatin is replaced by solid gelatin. This is due to the fact that it was necessary to let the suspensions stand for at least 1 hour to allow the particles of powdered gelatin to swell before the viscosity measurements were made. During this time the liquid gelatin at or near the isoelectric point increases

rapidly in viscosity while this increase in viscosity is suppressed where the hydrogen ion concentration is higher. This is proved by Fig. 72 which gives the viscosity of the supernatant solutions of gelatin (without the suspended particles) which had been standing for 1 hour. Inside the range of pH 4.4 and 4.6 the viscosity had risen more rapidly on standing than at the lower pH; which means that at or near the isoelectric point new submicroscopic particles of solid jelly are constantly formed from the molecules while this process is the slower the higher the hydrogen ion concentration. While thus the addition of acid to a solution of isoelectric gelatin retards the rate of formation of new submicro-

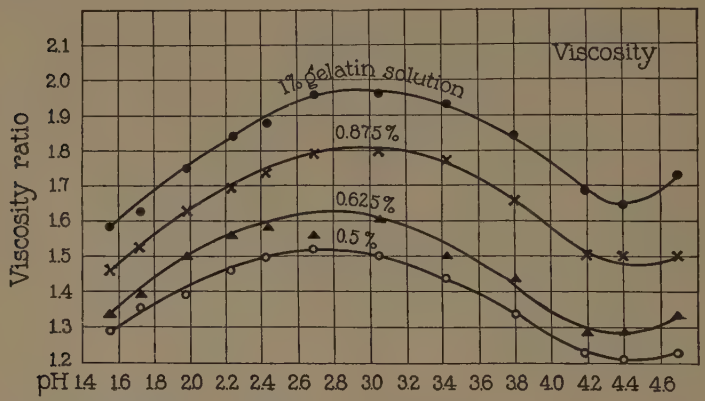

FIG. 72.—Viscosity of gelatin solutions after standing for 1 hour at 20°C. Notice minimum at pH 4.4, indicating that the viscosity has risen more near the isoelectric point on account of the formation of submicroscopic particles of gel.

scopic particles of jelly, it increases the swelling of those already present when the acid is added. On the other hand, powdered particles of isoelectric gelatin in water of pH 4.7 do not increase their volume on standing.

The fact that the addition of acid to a solution of isoelectric gelatin inhibits or retards the formation of new solid particles on standing was discussed in the preceding chapter.

If we now return to the discussion of the curves in Fig. 71 we may say that the results in that part of the curves which belongs to the abscissæ of pH above 3.8 is the expression of the fact that that part of the viscosity which is due to the gelatin in solution

had undergone an increase during the hour the solution had been standing at 20°C. after having been heated to 45°C.; and that the increase caused in the viscosity of the liquid gelatin was a maximum at the isoelectric point, being almost zero at a pH below 3.4; while the addition of acid had the opposite effect on the solid granules of gelatin, since their volume was increased according to the rules of the Donnan equilibrium.

It is necessary that we convince ourselves that a Donnan equilibrium exists when particles of solid gelatin are suspended in a solution of gelatin. That this is actually true was shown in the following way: 0.5-gm. doses of powdered gelatin were added to 100 c.c. of 0.5 per cent gelatin chloride solutions of different pH. The different beakers containing these mixtures were kept for 3½ hours at 20°C. The mass was then filtered through cotton wool and the pH of the filtrate (0.5 per cent gelatin solution) and of the solid gelatin granules were determined, that of the latter after they had been melted. It was found that the pH of the gelatin granules was higher than that of the solution and that the difference followed the Donnan equilibrium equation (Table XLIV), though the result was slightly irregular owing to the fact that it is impossible to free the suspended particles of gelatin completely from the supernatant liquid. When we separate a gelatin solution from water by a collodion membrane we have two equilibria, one across the membrane caused by the protein ions on one side of the membrane; and a second one inside the protein solution caused by the solid particles of jelly.

TABLE XLIV

pH of gelatin in suspension.....	5.12	4.60	4.49	4.18	4.07	3.73	3.45	3.93	2.68	2.34	2.09	1.86	1.77	1.53
pH of gelatin in solution.....	4.98	4.35	4.12	3.91	3.69	3.50	3.14	2.78	2.50	2.28	1.97	1.86	1.72	1.57

The reciprocal relation between viscosity and osmotic pressure of protein solutions disposes of the attempt to explain the influence of electrolytes on the physical properties of protein solutions on the basis of the micella or aggregate theory. We have seen that both the osmotic pressure of a gelatin chloride solution as well as its viscosity are depressed when a neutral salt is added

to the solution. The micella theory would explain this by assuming that the addition of a salt increases the degree of aggregation in the solution and hence diminishes the number of isolated particles and therefore the osmotic pressure of the solution. This assumption cannot be put to a quantitative test since we have no direct method of determining the number of aggregates in solution. We have shown, however, in this chapter that if we increase the number of aggregates at the expense of isolated protein ions or molecules, the viscosity rises. Hence, if we assume that the number of aggregates in the gelatin chloride solution is increased through the addition of salt, the viscosity of such a solution should increase for the same reason; whereas in reality we have seen that the addition of salt depresses both the osmotic pressure and the viscosity of the gelatin solution. This fact eliminates the aggregation or micella theory as a possible source of explanation of the colloidal behavior. We need not deplore the loss, since the application of the aggregate theory to the explanation of colloidal phenomena has never risen beyond the stage of vague speculations.

CHAPTER XIV

THE STABILITY OF PROTEIN SOLUTIONS[1]

A. The Stability of Aqueous and Alcoholic Solutions of Gelatin

1. It is difficult to discuss the problem of the stability of colloidal solutions satisfactorily as long as we do not possess a complete theory of the solution of crystalloids. In a general way we can say that there seem to exist two different kinds of forces by which substances can be kept in solution, first, the general forces active in all solutions and which are supposed to be the forces of attraction between solvent and solute; and second, the special forces such as the mutual repulsion of the particles due to electrical charges. These latter special forces are supposed to become of significance only when the general forces of attraction between solute and solvent are comparatively feeble.

It was noticed long ago that colloids in general and proteins in particular behave very differently in regard to the concentration of salt required for precipitation, some requiring very high concentrations of salt for this purpose and others comparatively low concentrations.[2] There is apparently no transition between the two extremes. It was formerly believed that these differences in the concentration of salts required could be used for the classification of colloids, and some authors divide the proteins or colloids in general into two groups, those which exist in the form of suspensions ("suspensoids," "lyophobic" or "hydrophobic" colloids), and those which exist in the form of solutions ("emulsoids," "lyophilic" or "hydrophilic" colloids). The former are precipitated by low concentrations, the latter only by high concentrations of salt. It is of more interest to know the reason why the precipitation of one type requires high and of the other

[1] Loeb, J., and Loeb, R. F., *J. Gen. Physiol.*, vol. 4, p. 187, 1921–22.
[2] Hardy, W. B., *J. Physiol.*, vol. 33, p. 251, 1905–06.

low concentrations of salts than to invent names for the two cases.

We intend to show that where low concentrations of electrolytes are required for precipitation, the precipitating influence of the salt has the earmarks of the Donnan effect, inasmuch as the effective ion of the salt has the opposite sign of charge to that of the protein particles, while where high concentrations of salts are required no such relation exists; and we conclude from this that where low concentrations of salts suffice for precipitation, the precipitation is due to the diminution of the value (pH inside the colloidal particles minus pH of the outside solution), as shown in the chapter on the charge of colloidal particles, while where high concentrations of salts are required the precipitating influence is due to some other cause, possibly the weakening of the forces of attraction between protein molecules and the molecules of the solvent, *e.g.*, water, through the presence of the salt. This latter conclusion, of course, would imply that the proteins can exist in true crystalloidal solution, the ultimate units being protein molecules or ions. There is no proof against the permissibility of such an assumption in the case of aqueous solutions of crystalline egg albumin (at the proper temperature, pH, and concentration) or of gelatin. This opinion is shared by Sørensen, who has not hesitated to determine the molecular weight of crystalline egg albumin from the osmotic pressure of its solution.[1]

2. Solutions of gelatin in water require enormous concentrations of salts for precipitation, and this process of salting out has no connection with the Donnan equilibrium, since solutions of gelatin are more readily salted out by sulphates than by chlorides regardless of the pH of the protein solution. The same is true for solutions of crystalline egg albumin of pH 4.7 or above; when, however, the pH of the crystalline egg albumin becomes too low (*e.g.*, 2.0 or less), lower concentrations of salts will cause precipitation. The reason for this influence of the pH is mentioned at the end of this chapter.

Eight-tenths per cent solutions of gelatin were prepared at three different pH, namely 4.7 (isoelectric gelatin), 3.8 (gelatin chloride), and 6.4 to 7.0 (Na gelatinate). The purpose was to

[1] SØRENSEN, S. P. L., Studies on proteins; *Compt. rend. trav. Lab. Carlsberg*, vol. 12, Copenhagen, 1915–17.

find the molecular concentration of different salts—namely, $(NH_4)_2SO_4$, Na_2SO_4, $MgSO_4$, KCl, and $MgCl_2$—required for precipitation. Table XLV shows that regardless of the pH the sulphates are better precipitants than the chlorides. Wherever we are dealing with colloidal phenomena, *i.e.*, phenomena regulated by the Donnan equilibrium, we must expect that sulphates will have a more depressing effect than chlorides when the protein is on the acid side of the isoelectric point but no when it is on the alkaline side or at the isoelectric point. But this is not true for the influence of ions on the salting out of gelatin in aqueous solution.

TABLE XLV.—MINIMAL MOLAR CONCENTRATIONS REQUIRED TO PRECIPITATE 0.8 PER CENT SOLUTIONS OF GELATIN

pH of gelatin solution	Approximate molecular concentration of salt required for precipitation				
	$(NH_4)_2SO_4$	Na_2SO_4	$MgSO_4$	KCl	$MgCl_2$
4.7	15/16 M	6/8 M	10/8 M	> 3 M	> 3 M
3.8 (gelatin chloride)	13/16 M	5/8 M	7/8 M	3 M	> 3 M
6.4 to 7.0 (Na gelatinate)	16/16 M	7/8 M	9/8 M	> 3 M	> 3 M

The question arises, How does it happen that sulphates are better precipitants than chlorides? Some light is thrown on this fact by experiments on the rate of solution.

Powdered gelatin of not too small a size of grain (going through sieve 30 but not through sieve 60) was rendered isoelectric in the way described in Chap. II and about 0.8 gm. was put into 100 c.c. of each of a series of solutions of NaCl, $CaCl_2$, or Na_2SO_4, varying in concentration from M/4,096 to 2 M. The suspensions of the powdered gelatin were frequently stirred and the time required to practically completely dissolve all the grains of powdered gelatin in suspension at 35°C. was measured. The ordinates in Fig. 73 are the solution times of isoelectric gelatin, and the abscissæ are the molecular concentrations of the salt used. It is obvious that NaCl and still more $CaCl_2$ increase the rate of solution of isoelectric gelatin in water, and the more the higher the concentration of the salts added. There exists, however, a striking discontinuity in the Na_2SO_4 curve. As long

as the concentration of Na_2SO_4 is below M/32 it increases the solubility of gelatin, and the more so the higher the concentration. When, however, the concentration of Na_2SO_4 is above M/32, a further increase in the concentration of Na_2SO_4 diminishes the solubility of gelatin and the more so the higher the concentration of Na_2SO_4. $(NH_4)_2SO_4$ acts like Na_2SO_4. We now understand

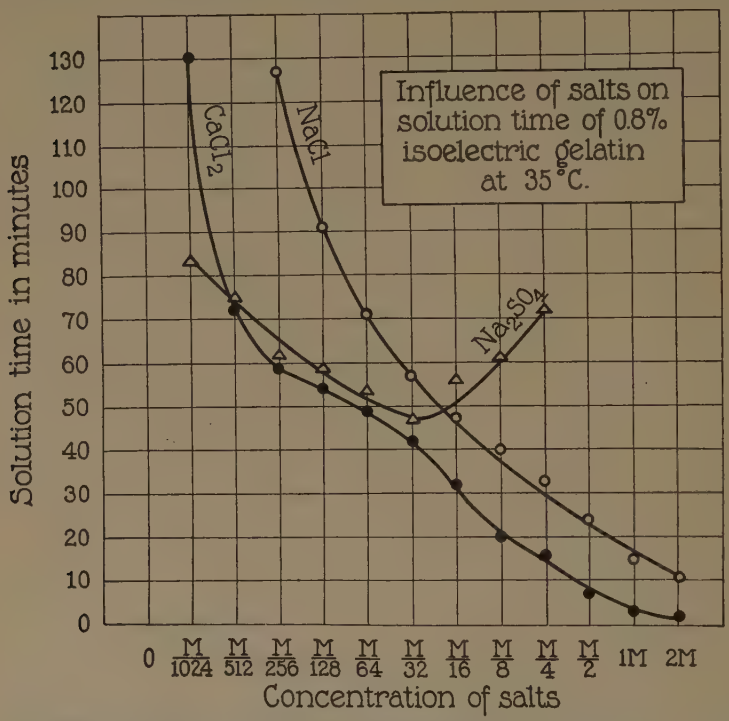

FIG. 73.—Influence of salts on the time required for the solution of 0.8 gm. of powdered isoelectric gelatin in 100 c.c. salt solution at 35°C. and pH 4.7. Notice difference of curve for Na_2SO_4 and for $CaCl_2$ and NaCl.

why we cannot precipitate solutions of isoelectric gelatin with KCl or $MgCl_2$ in concentrations up to 3 M (see Table XLV) while we can precipitate such solutions with sulphates but only at concentrations above M/2.

It may be of interest to supplement these observations by the results given in Table XLV*a*, on the influence of salt solutions of three different molar concentrations, namely, M/1,024, M/512,

and M/256, on the time required to dissolve 0.8 gm. of powdered isoelectric gelatin at 35°C. The salt solutions had a pH of 4.7. It is obvious from the table that the dissolving power of the chlorides increases with the valency of the cation while the dissolving power of the Na salts increases with the valency of the anion.

TABLE XLV*a*.—TIME IN MINUTES REQUIRED TO DISSOLVE 0.8 GM. OF POWDERED ISOELECTRIC GELATIN AT 35°C.

	M/256	M/512	M/1,024
LiCl	57	70	76
NaCl	49	66	75
KCl	56	70	80
$MgCl_2$	32	40	61
$CaCl_2$	32	40	62
$BaCl_2$	31	46	66
$CeCl_2$	26	35	44
$LaCl_3$	23		
Na_2SO_4	34	46	60
$Na_4Fe(CN)_6$	24	32	41

While isoelectric gelatin is only sparingly soluble, gelatin salts are highly soluble. Doses of 0.8 gm. of powdered gelatin of pH of about 3.3 dissolve very rapidly in 100 c.c. HCl of the same pH at 35°C. The addition of NaCl or $CaCl_2$ no longer increases the solubility, except for $CaCl_2$ in concentrations above M/16. Na_2SO_4 or $(NH_4)_2SO_4$ abruptly diminishes the solubility at a concentration above M/4; and NaCl does so above a concentration of 1 M (Fig. 74).

Figure 75 shows the influence of the three salts on the solution time of Na gelatinate of pH 10.5. Na_2SO_4 diminishes the solubility abruptly at a concentration above M/8 while both NaCl and $CaCl_2$ increase the solubility of Na gelatinate, NaCl in concentrations above M/2, and $CaCl_2$ in concentrations above M/16.

In all three cases, therefore, is the solubility of gelatin diminished by sulphates, but only exceptionally by chlorides. This explains the results contained in Table XLV. The solubility of gelatin in water does not depend on the Donnan equilibrium.

This conclusion is supported by an investigation of the influence of the pH on the solubility of gelatin.

We have seen that addition of little acid to isoelectric gelatin increases the osmotic pressure, viscosity, P.D., and swelling, while beyond a certain pH the addition of more acid has a depress-

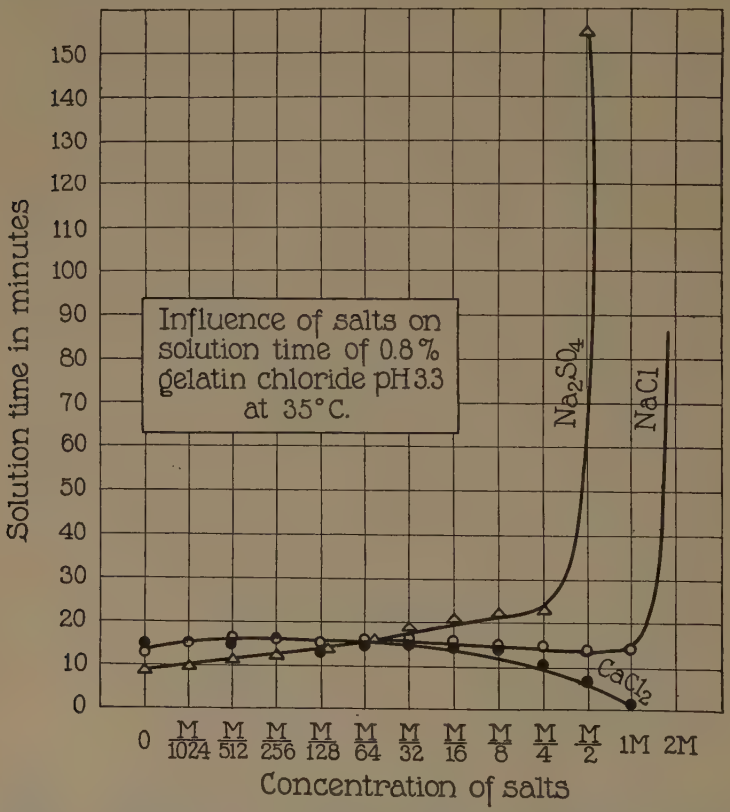

FIG. 74.—Influence of salt on solution time of 0.8 gm. of powdered gelatin chloride of pH 3.3 in 100 c.c. salt solution at pH 3.3. The gelatin is no longer soluble beyond 1M NaCl.

ing effect. It was of interest to find out whether such a maximum followed by a drop existed in the influence of acid on the solubility of gelatin. This is not the case at least between pH 4.7 and 1.0, since the solubility increases steadily with increasing hydrogen ion concentration, as was proven by measurements of

the dry weight of gelatin dissolved in a certain time at different pH. This corroborates the conclusion that the solution (and precipitation) of gelatin in water is not influenced by forces governed by the Donnan equilibrium and does therefore not show the characteristics of colloidal behavior. We are probably dealing in this case with forces of residual valency between water

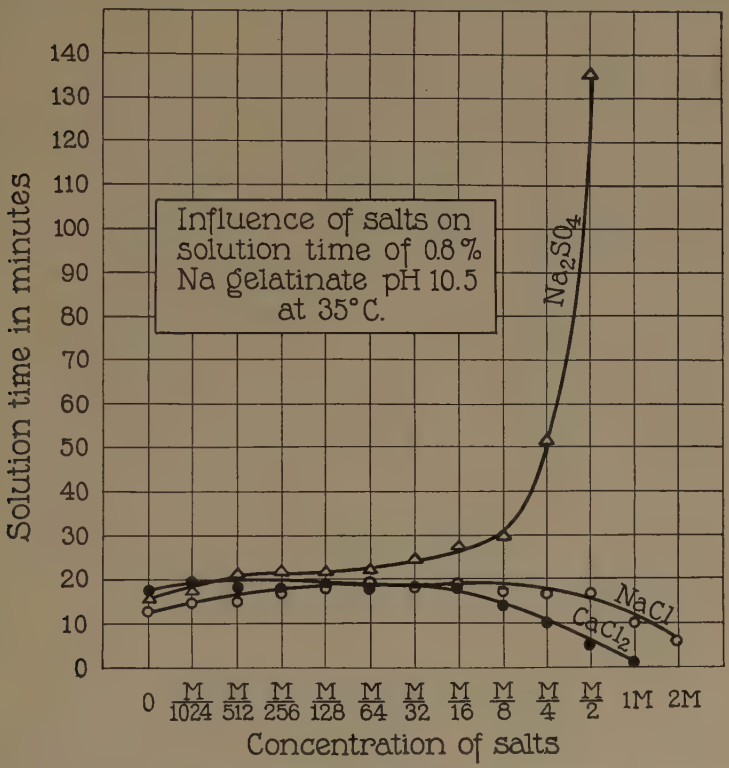

FIG. 75.—Influence of salts on solution time of 0.8 gm. of powdered Na gelatinate in 100 c.c. salt solution at pH 10.5.

and gelatin molecules, these forces being increased, as a rule, by the addition of salt to water, with the exception of the sulphates, which diminish the forces when added beyond a certain concentration at which the chlorides do not cause a diminution in solubility. This explains why it is easier to salt out gelatin from its aqueous solutions by sulphates than by chlorides.

These experiments also show that the solution of solid gelatin does not depend upon swelling (while the solution of casein chloride is, as we shall see, determined by swelling). The swelling of gelatin in acid reaches a maximum at pH of about 2.8 and then diminishes upon further increase in hydrogen ion concentration, while the rate of solution of solid gelatin granules continues to increase steadily when the hydrogen ion concentration increases beyond pH of 2.8 down to pH 1.0 (and possibly less). The mechanism of swelling and the mechanism of solution of solid gelatin in solutions of acid or alkali are determined by forces of an entirely different character; the swelling by osmotic pressure, and the solution in all probability by those forces which are responsible for the solution of crystalloids. The role of secondary valency forces in the process of solution is suggested by the following quotation from Langmuir.

"Acetic acid is readily soluble in water because the COOH group has a strong secondary valency by which it combines with water. Oleic acid is not soluble because the affinity of the hydrocarbon chains for water is less than their affinity for each other. When oleic acid is placed on water the acid spreads upon the water because by so doing *the COOH can dissolve in the water* without separating the hydrocarbon chains from each other.

"When the surface on which the acid spreads is sufficiently large the double bond in the hydrocarbon chain is also drawn onto the water surface, so that the area occupied is much greater than in the case of the saturated fatty acids.

"Oils which do not contain active groups, as for example pure paraffin oil, do not spread upon the surface of water."[1]

It should be added that if we replace the H in the carboxyl group of oleic acid by K the very soluble potassium oleate is formed, so that the whole molecule is now dragged into the water. The Na oleate is less soluble than K oleate. Ca oleate is again sparingly soluble.

In the case of proteins we have to deal with hydrocarbon groups possessing more affinity for each other than for water, and with COOH and NH_2 groups (or $CO\overline{O}$ and NH_3^+ groups) with a strong affinity for water. It is probable that the NH_2 or

[1] LANGMUIR, I., *J. Am. Chem. Soc.*, vol. 39, p. 1850, 1917.

NH_3^+ groups of the protein molecule are more active on the acid side of the isoelectric point and the COOH or $CO\bar{O}$ groups on the alkaline side of the isoelectric point. The analogy with the soaps would also suggest that the nature of the non-protein ion is of importance for the solubility of a protein salt. This is found to be true especially in the case of casein-acid salts, casein chloride being more soluble than casein nitrate, and the latter more soluble than casein trichloracetate.

Until evidence to the contrary is furnished, we must consider the possibility that the forces keeping proteins, such as gelatin or crystalline egg albumin, in aqueous solutions are the same forces which keep crystalloids in solution. The fact that gelatin solutions set to a gel does not necessarily contradict this conclusion. When gelatin solutions approach the gel state, (*i.e.*, when they reach a high viscosity), the relative distance of the protein molecules or ions from each other remains the same and the affinity of the active groups of the protein ions or molecules for water is not changed. The concentration of salt required for precipitation remains also practically the same.

3. At the isoelectric point the affinity of certain groups of the gelatin molecule for water is a relative minimum, as is shown by the fact that on standing at not too high a temperature, a 1 per cent solution of isoelectric gelatin will become cloudy and the suspended matter will settle; while this will not happen when the pH is either above 4.8 or below 4.6. When a little alcohol is added to such a solution near the isoelectric point, a rapid precipitation of the gelatin occurs. As soon as, however, the pH is 4.4 or below, or 5.0 or above, the gelatin in solution remains soluble even with an excess of alcohol, provided the anion of the acid or the cation of the alkali added to the isoelectric gelatin is monovalent (in the range of pH concerned), *e.g.*, Cl, CH_2COO, H_2PO_4, HC_2O_4, etc., or Li, Na, K, NH_4. When, however, these ions are bivalent, *e.g.*, SO_4, Ca, Ba, the solubility of the gelatin in alcohol is much less and the addition of a relatively small amount of alcohol will cause precipitation of the gelatin.[1] The addition of alcohol diminished the attraction between the watery groups of the gelatin molecule or ion and the solvent, and where these forces are small, as *e.g.* at the isoelectric point, or when the

[1] Loeb, J., *J. Gen. Physiol.*, vol. 3, p. 257, 1920–21.

ion in combination with the gelatin is bivalent, comparatively little alcohol suffices for precipitation regardless of the pH.

On the other hand, the addition of acid with monovalent anion or alkali with monovalent cation to isoelectric gelatin, so that the pH is either 4.4 or below or 5.0 or above increases the power of attraction between gelatin and water to such an extent that now the 1 per cent solution of originally isoelectric gelatin becomes soluble even when comparatively much alcohol is added.

Isoelectric crystalline egg albumin remains clear in solutions at low temperature, *e.g.*, 2°C., for many months even in a concentration of 8 per cent. When the temperature is raised, a change occurs in the molecule whereby its attraction for molecules of water is diminished and a 1 per cent solution precipitates at pH 4.8 at a temperature not far from 60°C. (the exact temperature was not ascertained). This precipitation is spoken of as the heat coagulation of egg albumin. If we add slight quantities of HCl the temperature at which the coagulation occurs is raised. At pH 4.39 the coagulation occurs on rapid heating at about 80°; at pH 4.25 or below the forces of attraction between the molecules of albumin and water become so great that heat coagulation no longer occurs even at 95°C.; the solution only becomes opalescent. It was found that the pH at which heat coagulation of a 1 per cent solution of crystalline egg albumin no longer occurs at 95°C. is approximately the same for HCl, HBr, HNO_3, CH_3COOH, H_3PO_4, and succinic acid. For oxalic and tartaric acids it is only slightly lower, probably because at this pH some of the acid anions are bivalent. The main fact is, that for H_2SO_4, whose anions are all bivalent, the pH at which coagulation becomes impossible is markedly lower; namely, 3.42. All this is in harmony with the writer's observations on the effect of different acids on the solubility of gelatin in alcohol-water mixtures.

On the alkaline side from the isoelectric point the critical pH at which heat coagulation disappears is practically identical for KOH and NaOH while the pH is considerably higher for $Ba(OH)_2$.[1]

The explanation of these phenomena is a part of the general problem of solubility; they have no direct connection with the theory of colloidal behavior.

[1] Unpublished results.

4. When some of the water of a gelatin chloride or Na gelatinate solution is replaced by ethyl alcohol, the mechanism which keeps the gelatin in solution is not changed, but when we continue increasing the relative amount of alcohol in the solution a critical point is reached where the amount of salt required for the precipitation changes abruptly.[1] We must conclude that the mechanism by which the gelatin is held in solution changes at or near this critical alcohol concentration. It is possible to show that when the amount of alcohol exceeds the critical limit the forces guaranteeing the stability of the solution of gelatin in the alcohol-water mixture are the forces resulting from a Donnan equilibrium.

We will first show that such a critical point exists for the ratio water: alcohol. Ten per cent solutions of gelatin chloride of pH 3.0 or of Na gelatinate of pH 10.0 were prepared. Five cubic centimeters of such a solution were first warmed to liquefy the gelatin and then while still warm they were diluted with 45 c.c. of a mixture of alcohol and water; the relative quantity of alcohol and water in the 45 c.c. varying. Ten cubic centimeters of these 1 per cent solutions of gelatin chloride or Na gelatinate in water-alcohol were titrated with a solution of a neutral salt, $(NH_4)_2SO_4$, NaCl, and $CaCl_2$, at 20°C. until precipitation occurred. It was noticeable that while it was not possible to precipitate the gelatin at all with 2½ M $CaCl_2$ or 5 M NaCl and only with comparatively high concentrations of $(NH_4)_2SO_4$ as long as the concentration of alcohol did not exceed a certain critical value, when this critical limit was exceeded traces of these salts sufficed for precipitation. This is illustrated in Tables XLVI and XLVII. When the solution contained no alcohol, *i.e.*, when 45 c.c. of H_2O were added to 5 c.c. of the 10 per cent solution of gelatin chloride of pH 3.0, 7.1 c.c. of 2 M $(NH_4)_2SO_4$ were required to cause precipitation (Table XLVI) in 10 c.c. of the 1 per cent gelatin chloride solution, and the quantity of $(NH_4)_2SO_4$ required increased at first the more H_2O was replaced by alcohol. When the 45 c.c. of liquid added to the 5 c.c. of 10 per cent gelatin solution consisted of 18.75 c.c. of water and 26.25 c.c. of ethyl alcohol, 17.8 c.c. of 2 M $(NH_4)_2SO_4$ were required to cause precipitation in 10 c.c. of the gelatin-alcohol-water mixture, but if now the proportion of

[1] The rest of this chapter is based on experiments not yet published.

TABLE XLVI.—CUBIC CENTIMETERS OF DIFFERENT SALT SOLUTIONS REQUIRED FOR FLOCCULATION OF GELATIN CHLORIDE, pH 3.0, IN DIFFERENT PROPORTIONS OF ALCOHOL AND WATER

Cubic centimeters of H_2O	45.0	40.0	30.0	25.0	20.0	18.75	17.5	15.0	10.0	8.75	7.5	6.75	5.0	0.0
Cubic centimeters of absolute alcohol	0.0	5.0	15.0	20.0	25.0	26.25	27.5	30.0	35.0	36.25	37.5	38.25	40.0	45.0
Cubic centimeters 2 M $(NH_4)_2SO_4$	7.1	9.5	11.6	12.1	15.2	17.8	**0.04**	**0.03**	**0.03**	.....	.....	.....	**0.03**	**0.02**
Cubic centimeters 5 M NaCl	7.8	13.7	20.4	36.4	49.0	.....	.....	∞	∞	∞	**0.2**			
Cubic centimeters 2½ M $CaCl_2$	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	**0.15**	**0.03**

TABLE XLVII.—CUBIC CENTIMETERS OF DIFFERENT SALT SOLUTIONS REQUIRED FOR FLOCCULATION OF Na GELATINATE, pH 10.0, IN DIFFERENT PROPORTIONS OF ALCOHOL AND WATER

Cubic centimeters of H_2O	45.0	40.0	35.0	30.0	25.0	22.5	21.25	20.0	15.0	13.75	12.5	10.0	5.0
Cubic centimeters of absolute alcohol	0.0	5.0	10.0	15.0	20.0	22.5	23.75	25.0	30.0	31.25	32.5	35.0	40.0
Cubic centimeters 2 M $(NH_4)_2SO_4$	11.0	16.0	18.5	20.4	23.7	....	.....	26.9	29.0	**1.5**	**0.7**	**0.03**	**0.02**
	light precipitate											heavy precipitate	
Cubic centimeters 5 M NaCl	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	**0.04**	**0.04**	**0.02**
Cubic centimeters 2½ M $CaCl_2$	∞	∞	∞	∞	∞	∞	∞	**0.03**	**0.02**	**0.02**	**0.02**		

alcohol to water was shifted only slightly in favor of alcohol, namely 17.5 c.c. of water and 27.5 c.c. of alcohol, 0.04 c.c. instead of 17.8 c.c. of 2 M $(NH_4)_2SO_4$ sufficed for precipitation (Table XLVI).

In the case of NaCl the drop was still more striking. When the 45 c.c. added to the 5 c.c. of 10 per cent gelatin solution consisted of 8.75 c.c. of water and 36.25 c.c. of alcohol, it was impossible to cause precipitation in 10 c.c. of the gelatin chloride-alcohol-water mixture with any amount of 5 M NaCl. When, however, the proportion of alcohol was only slightly increased, namely 7.5 c.c. of water and 37.5 c.c. of alcohol, 0.2 c.c. of NaCl sufficed for precipitation. In the case of $CaCl_2$ the critical point was reached when the ratio was 5 c.c. of water and 40 c.c. of alcohol.

The existence of the critical point can equally well be demonstrated in the case of Na gelatinate as is shown in Table XLVII.

What interests us is the following fact. The mechanism by which gelatin chloride of pH 3.0 and Na gelatinate of pH 10.0 are kept in solution is not altered as long as not too much of the water is replaced by alcohol, since in this case $(NH_4)_2SO_4$ is always a better precipitant than $CaCl_2$ for both gelatin chloride and Na gelatinate. When, however, the relative amount of alcohol exceeds a certain critical point, the mechanism by which the particles of gelatin are held in solution changes abruptly as is indicated by two facts; namely, first that the concentration of the salt required for precipitation becomes suddenly very small, and second, that the efficient ion has now the opposite sign of charge to that of the colloidal particle. Thus, in the case of gelatin chloride (Table XLVI), the critical points for $CaCl_2$ and NaCl are close together while the critical point for $(NH_4)_2SO_4$ is at a much lower concentration of alcohol. In this case the gelatin ion has a positive charge and the precipitating ion on the alcohol side of the critical point is the anion. In Table XLVII the critical points for $(NH_4)_2SO_4$ and for NaCl are close together while the critical point for $CaCl_2$ lies at a much lower concentration of alcohol. In this case the colloidal ion is negatively charged and the precipitating ion of the salt is the cation. The valency effect will be demonstrated more strikingly in a later part of this chapter.

5. If the precipitating effect of low concentrations of neutral

salts on colloidal solutions or suspensions is the result of the Donnan equilibrium, the stability of such solutions must be due to the fact that when isolated protein ions are beginning to coalesce a Donnan equilibrium is set up between the solution inside each nascent micella and the outside solution, which results in a swelling of the nascent micella whereby the ions in the process of coalescence are forced apart again. This prevents the formation of new micellæ from protein ions as well as the coalescence into larger complexes of the micellæ already existing. Moreover, there must also originate a P.D. between the micella and the solution, and the mutual repulsion of the micellæ due to their electrification will also prevent the coalescence of individual micellæ into a precipitate. If a salt is added, the forces guaranteeing the stability of the colloidal solution, *e.g.*, the osmotic pressure, swelling, and P.D. of the micellæ are diminished. When these forces fall below a certain minimal value the protein particles will coalesce.

We have seen that the depressing effect of a salt on swelling, osmotic pressure, and P.D. of protein particles is due to that ion of the crystalloidal salt which has the opposite sign of charge to that of the protein ion; and that the depressing effect of this crystalloidal ion increases with its valency. Thus, Fig. 76 indicates the depressing effect of different concentrations of NaCl and Na_2SO_4 on osmotic pressure and P.D. of a 1 per cent gelatin chloride solution of pH of originally 3.5. The abscissæ are the concentration of the salt added, the ordinates the osmotic pressure and P.D. The figure shows that the depressing effect of the same molecular concentration of Na_2SO_4 is much more than twice as great as the depressing effect of NaCl. If we assume that the protein ions and protein micellæ can coalesce when the osmotic pressure is 100 mm. and the P.D. about 4 millivolts, this low osmotic pressure and low P.D. of the 1 per cent solution of gelatin chloride of pH originally 3.5 will be produced when the NaCl solution is about M/64 and the Na_2SO_4 about M/512. The precipitating effect of Na_2SO_4 on gelatin chloride would then be about eight times as great as the precipitating effect of NaCl. The depressing effect of $CaCl_2$ on the osmotic pressure, swelling, and P.D. is about the same as that of a NaCl solution of the same concentration of chlorine ions, showing that the depressing effect

is due to the anion. Hence, the precipitating effect of $CaCl_2$ on gelatin chloride is about the same as that of a NaCl solution of the same concentration of Cl ions.

When the gelatin ion has a negative charge, *e.g.*, in the case of Na gelatinate, the depressing effect of neutral salts on the P.D., osmotic pressure, or swelling of the gelatin solution is due to the

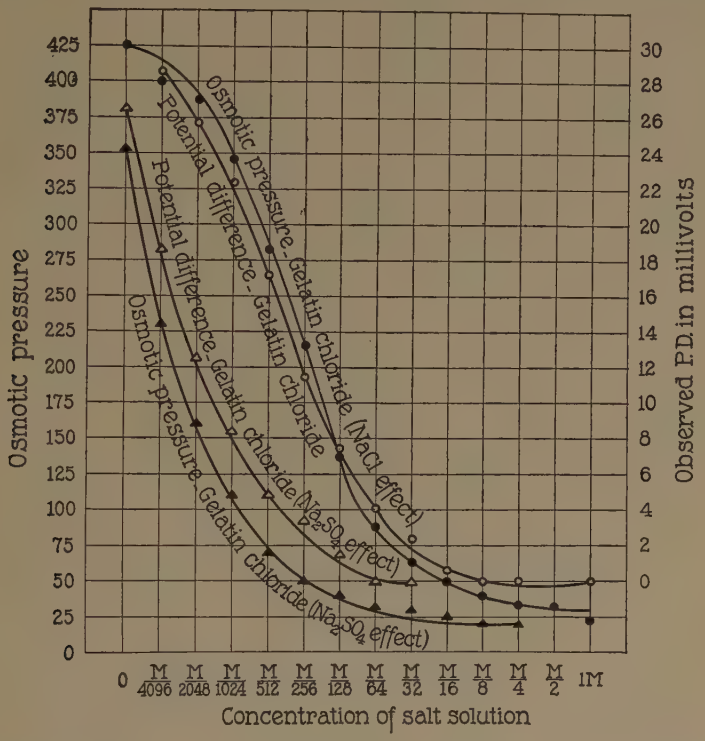

FIG. 76.—Depressing effect of salts (NaCl and Na_2SO_4) on P.D. and on osmotic pressure of a 1 per cent gelatin chloride solution of pH 3.5.

cation of the neutral salt and increases rapidly with the valency of the salt. This is illustrated in Fig. 37, p. 107, which expresses the effect of neutral salts on the swelling of Na gelatinate of a pH of about 9.3. It is obvious that in order to depress the original volume of the Na gelatinate to one-half a M/512 solution of $CaCl_2$ and a M/16 solution of NaCl and about M/32 solution of Na_2SO_4 are required. In other words, the depressing action of

$CaCl_2$ on the swelling of Na gelatinate is more than 10 times as great as that of NaCl. While these data are only semiquantitative, they enable us to form an approximate estimate as to whether or not the precipitating action of a salt on gelatin can be due to a diminution of the osmotic pressure (or P.D.) between the coalescent ions of gelatin in conformity with the Donnan equilibrium.

The procedure in our experiments was as follows: A stock solution of 5 per cent gelatin chloride of pH 3.0 was prepared; 2 c.c. of this solution were heated to about 45°C. to bring about complete liquefaction, and 50 c.c. of absolute alcohol were added while the gelatin was still warm and liquid. This concentration of alcohol was in excess of that required for the critical limit, and the gelatin solution was slightly opalescent. Ten cubic centimeters of this mixture of gelatin-alcohol-water were titrated with different salt solutions until a precipitate was found. The concentration of the salt solution was selected in such a way that not less than 0.3 and not more than 0.8 c.c. of solution were required for precipitation to avoid the addition of too large a volume of water to the solution. The difference in the relative efficiency of the different electrolytes is therefore expressed chiefly in the concentration of the solution required for precipitation. The reader should bear in mind that the pH of the gelatin chloride solution after the alcohol and the salt solution were added could not be measured, and that it was probably higher than 3.0 and about the same in all solutions. In order to be able to compare the relative flocculating efficiency of different salts the flocculating concentration is expressed in equivalents of cubic centimeters of M/1,024.

Table XLVIII shows that all salts with monovalent anion have a lower flocculating power on gelatin chloride than salts with divalent anion.

Salts with monovalent anion require a molecular concentration of about 100/1,024, *i.e.*, about M/10 concentration for precipitation, while those of the second group require a molecular concentration of about 10/1,024, *i.e.*, about M/100 or less. This shows that the difference in the flocculating power of monovalent and bivalent anions has roughly the ratio of about 1:10, *i.e.*, that it corresponds to the ratio to be expected from Fig. 76 within the

limits of the accuracy of these experiments, which is not very great.

TABLE XLVIII.—FLOCCULATING CONCENTRATION OF DIFFERENT SALTS AND ACIDS IN AN ALCOHOL-WATER MIXTURE OF GELATIN CHLORIDE

Concentration of salt used	Nature of salt	Cubic centimeters of salt solution required	Equivalent, cubic centimeters M/1,024
M/8	RbCl	0.8	102.0
M/8	KCl	0.8	102.0
M/8	NaCl	0.8	102.0
M/8	LiCl	0.7	90.0
M/8	$MgCl_2$	0.5	64.0
M/8	$CaCl_2$	0.65	83.0
M/8	$SrCl_2$	0.60	77.0
M/4	$BaCl_2$	0.5	128.0
M/8	$LaCl_3$	0.7	90.0
M/2	$CeCl_3$	0.35	179.0
M/2	$AlCl_3$	0.3	153.0
M/4	HCl	0.4	102.0
M/8	NaBr	0.8	102.0
M/4	HBr	0.3	77.0
M/8	NaI	0.6	77.0
M/8	$NaNO_3$	0.7	90.0
M/4	HNO_3	0.3	77.0
M/8	NaCNS	0.4	51.0
M/128	Na_2SO_4	0.8	6.4
M/32	H_2SO_4	0.35	11.2
M/64	Na_2 oxalate	0.65	10.4
M/128	Na_3 citrate	0.7	5.6
M/128	$Na_4Fe(CN)_6$	0.4	3.2

It was almost impossible to cause flocculation with acetic acid, oxalic acid, or tartaric acid. This suggests that secondary valency forces may play some role.

These experiments then show that the relative efficiency of Na_2SO_4 and NaCl for the flocculation of gelatin chloride in alcohol-water solution is apparently of about the same order of magnitude as their relative efficiency for the depression of the osmotic pressure of gelatin solutions.

Table XLIX shows the relative flocculating efficiency of cations on alcoholic solutions of Na gelatinate. Ten cubic centimeters of 1 per cent Na gelatinate of pH 10.0 were mixed with 50 c.c. of absolute alcohol. The mixture is slightly opalescent. Ten cubic centimeters of the mixture were titrated with various salt solutions until precipitation occurred.

TABLE XLIX.—FLOCCULATING CONCENTRATION OF DIFFERENT SALTS AND ALKALIES IN AN ALCOHOL-WATER MIXTURE OF Na GELATINATE

Concentration used	Nature of salt or alkali	Cubic centimeters of solution required	Equivalent, cubic centimeters M/1,024
M/16	NaCl	0.6	38.4
M/16	NaBr	0.6	38.4
M/16	NaI	0.45	29.0
M/16	$NaNO_3$	0.5	32.0
M/16	NaCNS	0.55	35.0
M/16	Na_2SO_4	0.75	48.0
M/16	Na_2 oxalate	0.6	38.4
M/16	Na_3 citrate	0.6	38.4
M/32	$Na_4Fe(CN)_6$	0.8	25.6
M/16	KCl	0.5	32.0
M/16	LiCl	0.6	38.4
M/4	KOH	0.3	77.0
M/4	NaOH	0.35	90.0
M/256	$MgCl_2$	0.55	2.2
M/512	$CaCl_2$	0.85	1.7
M/512	$SrCl_2$	0.8	1.6
M/512	$BaCl_2$	0.65	1.3
M/512	$LaCl_3$	0.6	1.2
M/512	$CeCl_3$	0.7	1.4
M/200	$Ca(OH)_2$	1.0	5.1
M/100	$Ba(OH)_2$	0.9	9.2

There are again two distinct groups, this time according to the valency of the cation. All electrolytes with monovalent cation require a molecular concentration of almost $\frac{40}{1,024} = \frac{1}{25}$ and all

salts with a cation of higher valency a concentration of almost M/500.

We, therefore, find that for the flocculation of Na gelatinate, originally of pH 10.0, salts with bivalent cation are about 20 times as efficient as salts with monovalent cation. This is roughly in harmony with the relative influence of these ions on the osmotic pressure of solutions of Na gelatinate, where the efficiency of a M/16 solution of NaCl is equaled by that of a M/512 solution of $CaCl_2$ (Fig. 37).

Schulze, Linder and Picton, and Hardy found that the precipitating ion has the opposite sign of charge to that of the colloidal particle and that the precipitating efficiency of the ion increases with its valency. These experiments suggest that the rule of Schulze, Linder and Picton, and Hardy is only a consequence of the Donnan equilibrium.

6. If the osmotic pressure set up between coalescing protein ions is able to prevent the formation of new micellæ and thus to contribute towards the stabilization of a solution of gelatin in a solution of much alcohol and little water, we can predict another result which will become clear from Fig. 77. This figure represents the influence of different concentrations of NaCl on the osmotic pressure of 1 per cent solutions of originally isoelectric gelatin brought to pH 1.8, 4.1, and 3.1 by the addition of different quantities of HCl. It is obvious from the curves that it requires a higher concentration of NaCl to bring the osmotic pressure of the gelatin chloride solution to the same low value, *e.g.*, 125 mm., when the pH is 3.1 than when it is 4.1. At pH 3.1 the concentration of NaCl must be between M/64 and M/128 and at pH 4.1 the concentration can be less than M/512. At pH 1.8 no addition of salt is required since the osmotic pressure is already below 125 mm. If it be true that the difference of osmotic pressure between the inside of the nascent micellæ and the surrounding solution is one of the forces guaranteeing the stability of the solution of gelatin in an alcohol-water mixture when the critical limit of alcohol is exceeded, it is obvious that the concentration of salt required for flocculation should vary with the original pH of the gelatin solution in the way characteristic for the Donnan equilibrium, namely that near the isoelectric point little or no salt should be required for precipitation, that with

increasing hydrogen ion concentration (*i.e.* increasing addition of HCl) the concentration of NaCl required for flocculation should first increase and later—after a certain pH—diminish. We will show that this is the case.

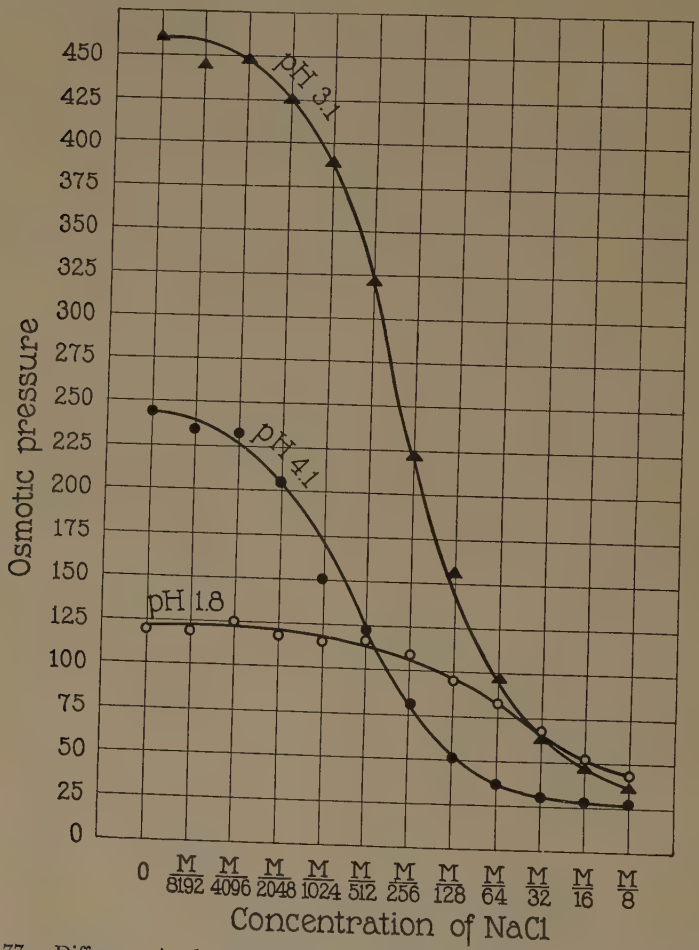

FIG. 77.—Difference in the depressing action of NaCl solutions on the osmotic pressure of gelatin chloride solution of different pH.

Ten cubic centimeters of a 5 per cent stock solution of isoelectric gelatin containing various amounts of HCl were brought to about 45°C. and 40 c.c. of absolute ethyl alcohol were added.

This was a quantity of alcohol in excess of that required to bring the solution to the critical point. After cooling to room temperature, the 50 c.c. of alcohol-water solution of 1 per cent originally isoelectric gelatin were titrated with a 2½ M NaCl or 2½ M $CaCl_2$ solution until permanent flocculation occurred. The number of cubic centimeters of 2½ M NaCl and $CaCl_2$ required varied with the pH of the original gelatin solution as Table L indicates. The pH in the table are those which the solution of gelatin would have had if it had been diluted with 40 c.c. of water instead of with alcohol. We do not know the actual pH in the alcoholic solutions except that it should be less than without alcohol.

Near the isoelectric point and in fact up to about pH 4.0 or 3.8 of the pH which would have been found had the solution contained no alcohol, the gelatin was not completely dissolved even when no salt was added, and the same was true when the pH (in our arbitrary standard) fell below 1.6. From pH 3.8 to pH 2.4 the cubic centimeters of 2½ M NaCl required for flocculation increased from 0.03 c.c. at pH 3.8 to 1.3 c.c. at pH 2.4; from then on it diminished again. Since the pH in the alcoholic solution was probably less than it would have been in a solution free from alcohol, the maximal stability of the gelatin in an alcohol-water mixture was at a pH greater than 2.4. These results are difficult to explain on any other basis than the Donnan equilibrium.

TABLE L.—CUBIC CENTIMETERS OF DIFFERENT SALT SOLUTIONS REQUIRED FOR FLOCCULATION OF GELATIN CHLORIDE AT DIFFERENT pH IN ALCOHOL-WATER MIXTURES

pH	3.8	3.4	3.3	3.1	3.0	2.82	2.7	2.5	2.4	2.3	2.2	2.0	1.9	1.8	1.7	1.65
Cubic centimeters 2½ M NaCl	0.03	0.03	0.05	0.15	0.6	0.75	1.0	1.2	1.3	1.25	1.0	0.9	0.75	0.45	0.40	0.25
Cubic centimeters 2½ M $CaCl_2$	trace	0.05	0.25	0.45	0.6	0.8	0.8	1.0	1.0	....	1.45	0.55	0.55	0.40	0.35	0.15
Gelatin chloride insoluble in the alcohol-water mixture below pH 3.8 and above 1.65																

We conclude from these experiments that gelatin forms a colloidal suspension in a mixture with much alcohol and little water and that the stability of the suspension depends in this case upon the forces set up by the Donnan equilibrium between the micellæ and the surrounding liquid, these forces being osmotic pressure and P.D.

In aqueous solutions or in solutions with little alcohol and much water the stability of the gelatin solution depends on forces which have no connection with the Donnan equilibrium and which may be the forces of secondary valency between gelatin ions or molecules and the molecules of water which are supposed to be responsible for the stability of crystalloidal solutions in general.

These forces of secondary valency do not cease to exist (though they are weak) in solutions with much alcohol and little water, and these forces may contribute also to the stability of the solution. This seems to be indicated by some of the data in Table XLVIII. This table shows that M/10 NaCl precipitates gelatin from the solution in much alcohol and little water. If the forces due to the Donnan equilibrium alone determine the stability of the suspension, a M/20 $CaCl_2$ solution and a M/30 $LaCl_3$ solution should have the same effect, since only the anion acts in the case of the Donnan effect when gelatin is positively charged. Table XLVIII shows that the $CaCl_2$ and $LaCl_3$ solutions required for precipitation are higher than M/20 or M/30, namely M/12 for $CaCl_2$ and M/11 for $LaCl_3$. This means that Ca and still more La have an inhibiting effect on the precipitation. We have seen that Ca and La increase the solubility of isoelectric gelatin in water, *i.e.*, they increase the forces of attraction between water and gelatin (see Table XLVIII). It is possible that the inhibition of the precipitating effect of Cl by La and Ca is due to the augmenting effect of these cations on the solubility of gelatin in water. This inhibiting effect on precipitation is often spoken of as the peptization effect. While the precipitating effect is due to the action of salts on the Donnan equilibrium, the peptization effect seems to be due to the influence of salts on the secondary valency forces between molecules of gelatin and solvent, in these experiments at least.

A few remarks may be added concerning the precipitation of crystalline egg albumin from aqueous solutions by salts at room

temperature. While the precipitation of solutions of sodium albuminate and of isoelectric albumin requires enormous concentrations of salts, the precipitation of solutions of albumin chloride of pH 2.0 or below is brought about by salt solutions of much lower concentrations (*e.g.*, M/2 NaCl, M/4 $MgCl_2$, etc.). This is, perhaps, connected with the fact that solutions of albumin chloride become opalescent at high hydrogen ion concentrations, and, therefore, assume more the character of suspensions.

The precipitation of albumin chloride by salts, from solutions in much alcohol and little water, gives results similar to those reported for the precipitation of gelatin chloride from solutions in much alcohol and little water. The inhibiting action of the divalent and trivalent cations was also observed in the case of the precipitation of albumin chloride from alcoholic solutions. The precipitation of Na albuminate by salts, from solutions in much alcohol and little water, gives results similar to those reported for the precipitation of Na gelatinate from solutions in much alcohol and little water. The alcoholic albumin solutions used were slightly opalescent, or in other words, they were no longer solutions but primarily suspensions of micellæ.

CHAPTER XV

THE STABILITY OF PROTEIN SOLUTIONS (*Continued*)

B. The Stability of Solutions of Casein in Water[1]

Since isoelectric casein is practically insoluble in water it is easy to study the mechanism of solution of granules of casein in aqueous solutions of acid and alkali. When this is done it is found that this mechanism is entirely different in the two media. In an alkaline solution, *e.g.*, NaOH, casein granules dissolve very much as do particles of sodium oleate, the solution of which is accompanied by phenomena of spreading. According to Quincke such phenomena of spreading are due to a sudden lowering of surface tension between the surface layer of soap and water, whereby projecting small particles of the surface are torn off so that the surface of the granules soon becomes smooth. This happens in the case of casein granules in alkali. There is no swelling noticeable in the particle.

The forces which drive the Na caseinate into solution are not the forces of the Donnan equilibrium. If this were the case the rate of solution of the granules should reach a maximum at a pH of between 10.0 and 12.0 and should then diminish. As a matter of fact the rapidity of solution increases indefinitely with the pH of the NaOH. In M/2 NaOH the solution of the granule occurs almost instantaneously. This agrees with the fact that solutions of Na caseinate in water require very high concentrations of NaCl or LiCl or NH_4Cl for precipitation.

A Na caseinate solution of pH 7.0 was prepared containing 2 gm. of originally isoelectric casein in 100 c.c. solution. Five cubic centimeters of this solution were added to 5 c.c. of solutions of different salts also of pH 7.0. No precipitation was observed when the concentration of NaCl in the caseinate solution was $2\frac{1}{2}$ M or that of LiCl was $3\frac{1}{4}$ M, or that of NH_4Cl was 2 M.

[1] Loeb, J., and Loeb, R. F., *J. Gen. Physiol.*, vol. 4, p. 187, 1921–22.

Precipitation occurred in $(NH_4)_2SO_4$ when the concentration of this salt in the casein solution was 2 M. Precipitation occurred in low concentrations of $CaCl_2$, namely M/128. In this latter respect the solution of Na caseinate differed from a solution of Na gelatinate in water. The facts indicate that the stability of a solution of Na caseinate in water is not due to a Donnan equilibrium.

It can be shown that the solution of granules of isoelectric casein in HCl depends on forces regulated by the Donnan equilibrium and that the rule of Hardy is only a consequence of this fact. This can be proven by microscopic observation of the mechanism of the solution of solid particles of originally isoelectric casein in solutions of acids of different concentration. It was found that the particles of casein swell in a solution of HCl, becoming more and more transparent the more they swell, and that when the swelling has reached a certain stage, the particles disappear—they are dissolved. When in the swollen stage, slight agitation may make them fall apart. T. B. Robertson had suggested such a mechanism for the solution of Na caseinate, but we have seen that the mechanism of solution in this latter case is different. There is no doubt, however, that the swelling of casein particles is a necessary prerequisite for the solution of casein-acid salts, since such particles are only dissolved when their swelling exceeds a definite limit.

The method of procedure was as follows: A small number of granules of isoelectric casein of the same size (going through a sieve with mesh 100 but not through a sieve with mesh 120) were put into 50 c.c. of water containing different quantities of different acids and kept at 24°C. At various intervals, *i.e.*, after 15, and 60 minutes, and 6, and 24 hours, the diameter of about 15 grains was measured with a micrometer under a microscope and the average diameter calculated. The particles were not stirred, and care was taken to avoid their breaking into smaller fragments. The averages after 1 hour are plotted in Fig. 78. The abscissæ are the logarithms of the concentrations of acid of the aqueous solution, the ordinates are the average diameters of the particles. It is obvious that the average diameter of the particles increases at first with the increase of the concentration of the acid, reaching a maximum at about pH

2.0 of the outside solution, and with a further increase in the concentration of the acid the swelling becomes less again.

Figure 79 gives the measurements of the same particles after 24 hours. At this time all the particles in the region of greatest solubility for HCl and for H_3PO_4, *i.e.*, between pH of the outside

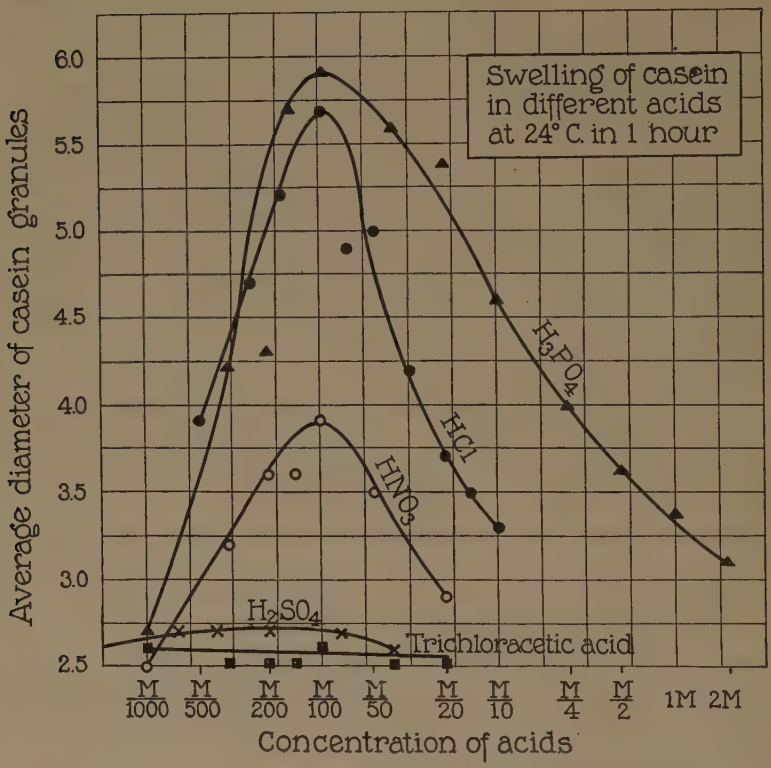

FIG. 78.—Influence of different acids on the swelling of casein.

solution of 1.8 and 2.9, had completely dissolved and could no longer be measured.

Figure 79 shows another fact; namely, that the rate of swelling is not the same in different acids. It is about the same in HCl and H_3PO_4 (for the same pH) but decidedly less in HNO_3 and still less in H_2SO_4 and trichloracetic acid. It was found that the rate of solution of casein in these different acids followed closely

the rate of swelling. It took longer to dissolve casein in HNO_3 than it did in HCl (at 20°C.); and the casein was practically insoluble in H_2SO_4 and trichloracetic acid in 24 hours.

The rate of swelling is a function apparently not only of the osmotic pressure inside the particle caused by the Donnan equilib-

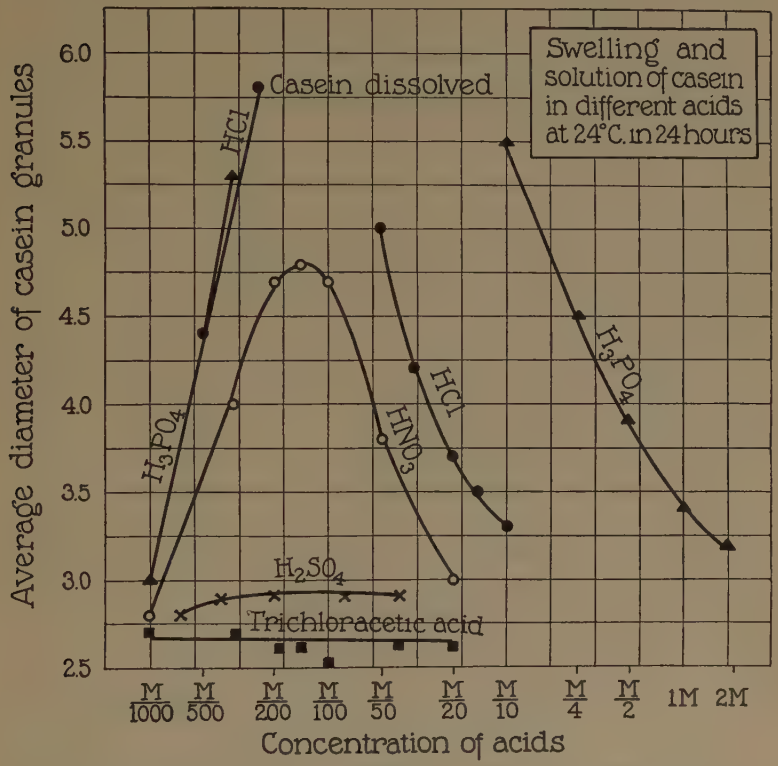

FIG. 79.—Connection between swelling and solution of casein particles.

rium, but also of the force of cohesion between the particles. Procter and Wilson have suggested that the rapid increase of swelling of solid gelatin with a rise in temperature is due to a corresponding diminution of cohesion between the molecules of gelatin with rising temperature. The influence of the anion of gelatin-acid salts on the cohesion of the particles of a solid gel is apparently much smaller than the influence of the anion on the

cohesion of particles of casein-acid salts. The forces of cohesion in the case of casein sulphate and casein trichloracetate seem to be so great that they cannot be overcome by the osmotic pressure due to the Donnan equilibrium; and hence, no swelling (and as a consequence no solution) of solid casein is possible in H_2SO_4 or trichloracetic acid. The influence of valency on the Donnan equilibrium is the same in the case of the swelling of casein and of

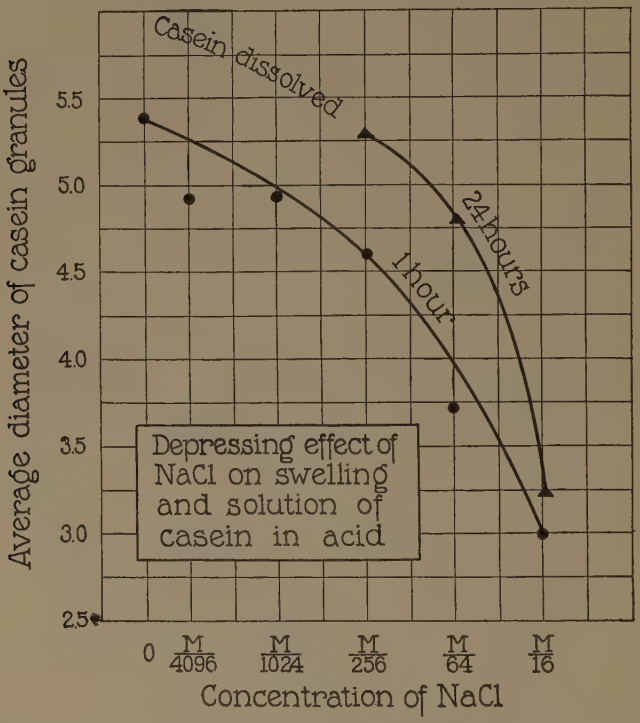

FIG. 80.—Depressing action of NaCl on swelling and solution of casein in acid.

gelatin; what is different is the influence of certain ions on the relative affinity of casein ions for water and for each other.

Procter and Wilson have shown that the theory of the Donnan equilibrium explains the depressing effect of a salt on the swelling of solid gelatin. Microscopic measurements of the influence of NaCl on the rate of swelling of individual grains of casein particles in M/100 HCl were made at 24°C., and the results plotted in

Fig. 80. The ordinates are the average diameters of the particles after 1 and 24 hours respectively. The abscissæ are the concentrations of NaCl. The depressing effect is similar to that found in the case of the swelling of a jelly of gelatin. After 24 hours the particles had dissolved in the NaCl solutions of a concentration below M/256, but not in concentrations of NaCl higher than M/256.

That the solution of casein chloride is thus regulated to a large extent by the Donnan effect was ascertained also by measurements of the quantity of casein chloride dissolved at 20°C. at various pH of the solution. One gram of isoelectric powdered casein was put into 100 c.c. of solutions of HCl of different concentration and kept in these solutions in one case for 1 hour, in a second case for 22 hours. The mass was then poured into graduated cylinders and the undissolved part was allowed to settle to the bottom for 2 and for 6 hours respectively at 20°C. The supernatant liquid was removed and the sediment dried over night in an oven at about 100°C. Table LI gives the result. The dry weight of 1 gm. of isoelectric casein was found to be 0.870 gm. and this weight diminished by the dry weight of the sediment was the amount dissolved. Table LI shows that the rate of solution increases with diminishing pH from 4.36 to 2.18 where the solubility of casein chloride is a maximum; with a further decline in pH the solubility diminishes again. This is in agreement with the Donnan effect.

In a similar way the depressing effect of NaCl on the rate of solution of casein chloride was ascertained (Fig. 80). Solutions of 12.5 c.c. of 0.1 N HCl in 100 c.c. and containing 1 gm. of powdered, originally

TABLE LI.—AMOUNT OF CASEIN DISSOLVED AT 20°C. IN HCl OF DIFFERENT pH

pH	4.36	3.32	3.11	2.97	2.94	2.84	2.75	2.64	2.53	2.36	2.18	2.06	1.87	1.66	1.50	1.40
Milligrams dissolved after 1 hour.....	42	55	86	249	265	....	....	348	408	....	547	538	401	366	272	219
Milligrams dissolved after 22 hours.....	102	133	164	267	342	459	536	634	646	733	788	779	710	528	374	300

isoelectric casein were prepared in 0, M/2,048, M/1,024, to M/4 NaCl. The pH of a solution of 1 gm. of casein in 100 c.c. containing 12.5 c.c. of 0.1 N HCl was 2.12 and this pH was the same in all solutions made up in NaCl. The solution was kept at 20°C. for 16 hours and then allowed to settle for 24 hours at 20° in 100 c.c. graduated cylinders. The dry weight of the sediment was determined and this weight when deducted from the dry weight of 1 gm. isoelectric casein, namely, 0.870 gm., was the amount that had gone into solution after a correction was made for the free NaCl held in 2 c.c. solution which was arbitrarily assumed not to have been removed. Though this latter correction was somewhat arbitrary, it could have caused a noticeable error only when the concentration of the salt solution exceeded M/64. For the solutions of M/64 and below this error was negligible. Table LII gives the number of milligrams of casein which had gone into solution.

TABLE LII

	Concentration of NaCl					
	M/2,048	M/1,024	M/512	M/256	M/128	M/64
Milligrams dissolved.....	714	685	665	615	449	282

The main fact is that a slight increase in the concentration of NaCl causes a noticeable drop in the rate of solution. Thus, M/1,024 NaCl causes a noticeable diminution in the solubility of a 1 per cent solution of casein chloride of pH 2.12 at 24°.

These observations then indicate that the solution of solid particles of casein chloride is brought about by the ultimate elements being forced apart mechanically through the process of swelling. The force acting in this swelling is the hydrostatic pressure of the water which is forced into the interstices of the solid particles by the osmotic pressure of the solution in the interstices between the casein ions. Procter and Wilson have shown that the application of Donnan's theory of membrane equilibrium accounts quantitatively for this swelling on the assumption that swelling is caused by the excess of the osmotic pressure inside the gel over that of the surrounding solution

(Chap. XI). As soon as the osmotic pressure in the particle exceeds the forces of cohesion between the casein ions of the particle, the casein ions constituting the particle are separated.

The question then arises, How can the Donnan effect stabilize the particles of casein chloride in solution, and how can we explain the precipitating effect of low concentrations of neutral salts? Let us assume that the ultimate particles in a solution of casein chloride of pH 2.2 are, (*a*) isolated casein ions, (*b*) isolated casein molecules, and (*c*) small casein aggregates or micellæ. The Donnan equilibrium furnishes two kinds of forces preventing that degree of coalescence of these particles which is required for precipitation; namely, the osmotic pressure and the membrane potentials. When isolated protein ions collide and remain attached to form a micella, a Donnan equilibrium is established between the nascent micella and the surrounding solution. The Donnan equilibrium demands that there be a higher concentration of electrolytes inside than outside and this difference in osmotic pressure leads to water being attracted into the micella. The increase in hydrostatic pressure will force the protein molecules apart again and thus tends to prevent the formation of the micellæ. Moreover, if micellæ exist in the casein chloride solution (aside from isolated casein ions and molecules) the coalescence of different micellæ into larger aggregates must be prevented by the potential difference between the micellæ and the surrounding solution. As a consequence of this P.D., the micellæ must repel each other. This charge as well as the osmotic pressure caused by the Donnan equilibrium is a minimum at the isoelectric point, rises rapidly with increasing hydrogen ion concentration, reaching a maximum, and diminishes again with a further increase in hydrogen ion concentration as shown in a preceding chapter. The osmotic pressure and charge are also diminished by the addition of salt. In this case, both the osmotic pressure as well as the P.D. are depressed, in accordance with Donnan's theory, and when this depression reaches a certain degree the casein particles coalesce. They will also aggregate without the addition of salt at or near the isoelectric point where these forces due to the Donnan equilibrium are also zero or sufficiently low.

These conclusions were supported by experiments on the precipitation of casein chloride solutions by salts. The concentra-

tions required should be comparatively low and this was found to be the case. One per cent solutions of casein chloride of pH 2.2 were prepared in different concentrations of salts in water of about the same pH. That concentration was determined which causes an almost instantaneous complete precipitation of the protein from the solution so that the supernatant liquid became as clear as water. These concentrations were as follows:

NaCl	about M/8
$NaNO_3$	about M/8
$CaCl_2$	about M/8
Na trichloracetate	about M/16
Na_2SO_4	about M/32

Though the results are only semi-quantitative, the validity of Hardy's rule and the valency effect are easily recognizable. It is also obvious that the concentrations of electrolytes required for instantaneous, complete precipitation of casein chloride are considerably lower than those required for the precipitation of Na caseinate from their watery solution.

Hardy's rule, that only that ion of a neutral salt is active in precipitation which has the opposite sign of charge to that of the colloidal ion, and that the efficiency of the ion increases with the valency, is simply the expression of the Donnan effect, as is also the fact that very low concentrations of electrolytes suffice for precipitation. The reader will notice that it is unnecessary to assume that the ions are adsorbed by the casein or that the adsorption of ions annihilates the electrical charges on the particles of casein.

CHAPTER XVII

COLLOIDAL SUBSTANCES, COLLOIDAL STATE, AND COLLOIDAL BEHAVIOR

Graham had suggested the distinction between colloidal and crystalloidal substances, but it was found later that one and the same substance, *e.g.*, NaCl, may behave when in solution either as a crystalloid or as a colloid. It then was proposed to drop the distinction between colloidal and crystalloidal *substances* and to distinguish between the colloidal and the crystalloidal *state of matter*. The reasons are summed up in the following quotation from Burton:

"Modern work has shown that it is incorrect to speak of colloidal substances as a particular class. Krafft has observed that the alkali salts of the higher fatty acids—stearate, palmitate, oleate—dissolve in alcohol as crystalloids with normal molecular weights, but in water they are true colloids. The reverse is true of sodium chloride; Paal found that the latter gave a colloidal solution in benzol, while, of course, it gives a crystalloidal solution in water (Karczag). More recently, von Weimarn has demonstrated, by the preparation of colloidal solutions of over two hundred chemical substances (salts, elements, etc.), that, by proper manipulation, almost any substance which exists in the solid state can be produced in solution, either as a colloid or as a crystalloid; and that, as shown by many other workers, in some cases it is merely a matter of the concentration of the reacting components whether one gets crystalloidal or colloidal solutions.

"Consequently, we now speak of matter being in the colloidal state rather than of certain substances as colloids—the essential characteristic of the colloidal state being that the substance will exist indefinitely as a suspension of solid (or, in some cases, probably liquid) masses of very small size in some liquid media, *e.g.*, water, alcohol, benzol, glycerine, etc. According to the medium employed the resulting solutions or suspensions are called, after Graham, hydrosols, alcosols, benzosols, glycersols, etc."[1]

[1] Burton, E. F., The Physical Properties of Colloidal Solutions, 2d ed., pp. 8–9, London, New York, Bombay, Calcutta, and Madras, 1921.

If we apply the conclusion drawn in this statement to the proteins, it follows that we have no longer any right to insist that proteins can form only colloidal solutions. If solutions of gelatin and of crystalline egg albumin behave like crystalloidal solutions in water and if gelatin solutions in alcohol behave like colloidal solutions, we have no right to say that nevertheless albumin and gelatin are in the colloidal state when dissolved in water. Such an assumption is as arbitrary as to say that NaCl is in a colloidal state when dissolved in water simply because it is in a colloidal state when dissolved in benzol. The idea that the two proteins mentioned must be in the colloidal state when dissolved in water is a survival from the time when it was customary to discriminate between colloidal and crystalloidal *substances* instead of between colloidal and crystalloidal states, and the terms emulsoids or hydrophilic colloids when applied to proteins which require high concentrations of salt for their precipitation are also a survival from that time. The fact that the molecules of protein are large does not matter, since the chemical constitution of solute and solvent and not the mere size of the molecules of solute determines the stability of their solution. The large size introduces only interesting complications inasmuch as it makes it possible that one and the same molecule may have groups with a different degree of attraction for the molecules of water or solvent.

In Burton's statement just quoted only one colloidal property is taken into consideration; namely, the stability of colloidal solutions. We have seen, however, that as far as the proteins are concerned there are a number of other properties of colloidal solutions which are all as characteristic for colloids as are the conditions for the stability of the solutions; and that as a matter of fact the stability of colloidal solutions depends on these other properties, such as the P.D. and the osmotic pressure. It is, therefore, no longer possible to base our definition of colloids exclusively on data derived from a study of the stability of colloidal solutions.

The general characteristics of colloidal behavior may be stated as follows:

1. Low concentrations of neutral salts depress the osmotic pressure, P.D., viscosity, and stability of colloidal solutions and the swelling of gels.

2. This depressing effect of the salt is always due to that ion which has the opposite sign of charge to that of the colloidal particle.

3. The depressing effect increases with the valency of the effective ion.

4. When the colloidal substance used is an amphoteric electrolyte the addition of little acid or alkali to the isoelectric substance increases the osmotic pressure, P.D., viscosity, stability of the solution, and swelling of gel until a point is reached where the further addition of acid or alkali will have the opposite effect.

5. The depressing effect of an excess of acid or alkali is also due to the ion which has the opposite sign of charge to that of the colloidal particle, and the efficiency of that ion also increases with its valency.

It is obvious that the stability of colloidal solutions is only one of a number of properties which all possess the same characteristic features. It has been shown in the preceding chapters that all these characteristics find their explanation in the Donnan equilibrium.

If, on the basis of this knowledge, we continue the mode of reasoning expressed in the quotation from Burton, we come to the further conclusion that it is no longer correct to discriminate between the colloidal and the crystalloidal state of matter, for the same substance may behave in the same state either like a colloid or a crystalloid. We have seen that a 1 per cent solution of crystalline egg albumin in water at room temperature and at a pH much above 1.0 will behave like a colloid or as if it were in the colloidal state in regard to osmotic pressure or in regard to P.D. when separated from water by a collodion membrane; but the same solution of crystalline egg albumin will behave like a crystalloid or as if it were in the crystalloidal state in regard to the stability of the solution and practically also in regard to viscosity (as long as the temperature and the concentration of the solution are not too high and the pH not too low). The reason for this is plain in the light of the preceding chapters. Proteins (and, perhaps, substances in general) will show colloidal behavior when the following two conditions are fulfilled: first, the substance must be capable of dissociating electrolytically, and second, one of the two oppositely charged ions must be prevented from

diffusing while the other is free to diffuse. In this case a Donnan equilibrium is established resulting in an unequal distribution of the diffusible ions on both sides of the membrane. The forces resulting from this distribution of crystalloidal ions are the only cause of those phenomena which are designated as colliodal.

In general the block in the diffusion of one kind of ions can be brought about by two kinds of conditions: first, by membranes with a selective permeability; and, second, by the cohesion between ions of one kind. An electrolyte which exists in a true solution (and this is in all probability true for crystalline egg albumin) will show colloidal behavior in regard to osmotic pressure and to P.D. when separated from pure water by a membrane which is permeable for all ions in the solution except one. Moreover, when ions of one type are attracted to each other with greater force than they are attracted by the molecules of the solvent, aggregates are formed, and when these aggregates are permeable to other ions than those forming the aggregate, a Donnan equilibrium is also established and colloidal behavior follows again. This latter condition leads to the colloidal character of swelling, viscosity, and of the stability of suspensions. Since 1 per cent solutions of crystalline egg albumin are very stable at room temperature and as long as the pH is not too low, there are few or practically no aggregates in such a solution, and the crystalline egg albumin behaves in regard to viscosity and in regard to the stability of its solutions essentially as if it were in the crystalloidal state. Of course, the proof has to be furnished that crystalline egg albumin exists in the form of isolated molecules in agneous solution. Sørensen has calculated the molecular weight of crystalline egg albumin from measurements of the osmotic pressure of this substance and has obtained results which make this conclusion probable.[1]

The behavior of gelatin is especially interesting. Gelatin solutions in water show colloidal behavior in regard to osmotic pressure and P.D. when the solutions are separated from pure water by a collodion membrane or by some other membrane with similar selective permeability. Gelatin solutions in water show

[1] SØRENSEN, S. P. L., Studies on proteins: *Compt. rend. trav. Lab. Carlsberg*, vol. 12, Copenhagen, 1915–17.

also colloidal behavior in regard to viscosity when the temperature is not too high, and this is due, as we have seen, to the existence in such solutions of submicroscopic particles of gel in which the diffusion of the protein ions is prevented by the forces of cohesion between the protein ions forming the gel. The degree of swelling, and the relative volume occupied by these particles in the solution is regulated by the Donnan equilibrium and, hence the viscosity of a gelatin solution is also regulated by the Donnan equilibrium; in other words, the viscosity of gelatin solutions has the peculiarities of colloidal behavior. Only in one respect do the aqueous solutions of gelatin behave as if gelatin were in the crystalloidal state, namely in respect to the stability of solutions. It requires high concentrations of salts to precipitate gelatin from its aquoues solutions and the sign of charge of the precipitating ion has no relation to the sign of charge of the protein ion. Of course, it remains still to be proved that gelatin exists in aqueous solution essentially in the form of isolated ions or molecules and almost exclusively so if the temperature is above 35°C. This proof can only be furnished if the calculations of the molecular weight from osmotic pressure measurements are supported by other measurements, especially by determinations of the chemical constitution of the gelatin molecule. Dakin's[1] analysis leads to a molecular weight which is quite compatible with the results from the osmotic pressure determinations (see Chap. X).

We have seen that solutions of gelatin in alcohol-water mixtures behave like suspensions inasmuch as they can be precipitated by low concentrations of salt and inasmuch as the precipitating ion has now the opposite sign of charge to that of the protein ion. When the gelatin solution is in this state, it differs in two respects from a gelatin solution in pure water: it has a comparatively low viscosity, and it no longer sets to a gel. It is also, as a rule, opalescent. The change in viscosity can be shown in the following way.[2]

To 1 gm. of isoelectric gelatin enough HCl is added so that in a 1 per cent solution in water the pH would be about 3.0. This gelatin is dissolved in mixtures of water and alcohol, heated rapidly to 45°C., and cooled rapidly to 15°C. The time of

[1] Dakin, H. D., *J. Biol. Chem.*, vol. 44, p. 499, 1920.

[2] The following experiments have not yet been published.

outflow through a viscometer is measured immediately at 15°C. As a control the time of outflow at 15°C. of identical water-alcohol mixtures but containing no gelatin is also measured. The ratio of the time of outflow of the gelatin-water-alcohol mixture to that of the water-alcohol mixture without gelatin, *i.e.*, the relative viscosity of the gelatin solution, is given in Table LIII. The upper horizontal row gives the relative amount of alcohol in per cent, the second row the appearance of the solution, the third the time of outflow of the gelatin solution in seconds, the fourth row the time of outflow of the alcohol-water mixture without gelatin, and the last row the relative viscosity of the

TABLE LIII.—INFLUENCE OF INCREASING QUANTITIES OF ALCOHOL ON THE VISCOSITY OF A 1 PER CENT SOLUTION OF GELATIN CHLORIDE OF ORIGINALLY pH 3.0

	Concentration of alcohol in per cent						
	0	40	70	80	85	87.5	90
Appearance of solution	clear				slightly opalescent	opalescent	very opalescent
Time of outflow of gelatin solution in seconds	207	266	506	362	229	185	163
Time of outflow of alcohol + water, without gelatin	80	233	225	194	178	168	160
Relative viscosity	2.590	2.860	2.250	1.860	1.286	1.100	1.020

gelatin solution. It is obvious that the viscosity drops sharply between 80 per cent and 85 per cent of alcohol, and that at 85 per cent. where the solution is already opalescent, the relative viscosity is only 1.286 and only 1.1 for 87.5 per cent alcohol.

In a second experiment the same solutions were prepared but the solutions were kept for 2 days in a thermostat at 9°C., the mixtures were then rapidly brought to 15°C., and the viscosities determined. The solution containing 60 per cent of alcohol or less had set to a jelly; the solution containing 70 per cent was almost solid, but the solutions containing 80 per cent or more were all completely liquid. Their relative viscosity was measured (Table LIV) and was found to be only slightly larger than at the beginning, when the solution contained 85 per cent or more

alcohol, while the viscosity had risen considerably when the solution contained less than 80 per cent alcohol.

The opalescence of the alcoholic solutions indicates the presence of aggregates of gelatin, but since the relative viscosity of these alcoholic solutions is low as compared with the viscosity of solutions of gelatin in pure water, and since the alcoholic solutions no longer set to a jelly, the micellæ in the watery solution and in the alcohol-water mixture, containing 80 per cent alcohol or more, must be different. The fact that the viscosity ratio is low in the opalescent gelatin-alcohol solutions (which no longer can set to a jelly) indicates that the micellæ in this solution

TABLE LIV.—VISCOSITY AT 15°C. AFTER THE SOLUTION HAD BEEN KEPT AT 9°C. FOR 2 DAYS

	Concentration of alcohol		
	80 per cent	85 per cent	90 per cent
Appearance of solution	slightly opalescent	opalescent	very opalescent
Time of outflow of gelatin solution in seconds	521.0	247.0	180.0
Time of outflow of alcohol-water mixture without gelatin	194.0	178.0	160.0
Relative viscosity of gelatin solution	2.685	1.390	1.125

occlude less water than the micellæ formed in the solutions of gelatin in water (or in water with not too much alcohol).

This is in harmony with the assumption (made in Chap. XIV) that the forces which hold gelatin in solution in pure water or in water with little alcohol, are different from those which hold the gelatin in solution when the critical limit for alcohol has been exceeded. In aqueous solutions or in solutions with much water and little alcohol where setting of gelatin to a jelly is possible, the molecules or ions of jelly are distributed evenly in the solvent probably on account of the strong forces of residual valency between solute and solvent. The large gelatin molecules can adhere to each other only through those

groups which have a stronger attraction for each other than they have for the solvent. The other groups adhere strongly to the solvent, and hence they cannot come in contact with each other even when the gelatin sets to a jelly. When a 1 per cent solution of gelatin sets to a gel the distribution of the gelatin molecules in the solvent undergoes probably no profound change. What may change is, perhaps, the orientation of the gelatin molecules or ions towards each other, but not their average distance from each other. When, however, too much alcohol is added, *i.e.*, when the solution is on the alcohol side of the critical point, the forces of attraction between gelatin and solvent are weakened to such an extent that the groups which were formerly attracted by the solvent are now more strongly attracted to each other than they are to the molecules of solvent. In the micellæ thus formed the protein ions or molecules are in much closer contact than they are in a jelly, and hence they occlude much less water than the micellæ formed in pure water or in mixtures of water with little alcohol. The latter micellæ increase the viscosity of the solution more than the micellæ formed in an excess of alcohol. The gelatin would be precipitated in the latter solutions if it were not for the fact that the coalescence of the protein ions and molecules is prevented by the forces set up as a consequence of the Donnan equilibrium, namely, forces of osmotic pressure and of P.D., as stated. When, however, a small quantity of salt is added the forces set up by the Donnan equilibrium are diminished and nothing now prevents the forces of attraction between the gelatin molecules from causing the separating out of the gelatin from solution. It should also be recalled that at the isoelectric point not only the P.D. but also the osmotic pressure of protein solutions is a minimum and that salts depress the osmotic pressure as well as the P.D.

The fact that a Donnan equilibrium is established between micellæ and surrounding liquid, whereby the opposite ions of electrolytes are distributed in a definite way between the two constituents, makes it clear why in the case of precipitation of colloids some of the precipitating electrolyte must be found in the precipitate, and that there can, as a rule, be no stoichiometric relation between the quantity of salt and the mass of protein in the precipitate.

We can form, on the basis of what has been said, a more definite picture of the difference between gel formation and precipitation. When gelatin is dissolved in pure water or in much water with little alcohol, probably only one of the groups of the gelatin molecule has a greater affinity for other like groups than for the molecules of water, while all the other groups of the gelatin molecules have much stronger affinities for water than for each other. Hence in this state the gelatin molecules can form networks by their "oily" group (*i.e.*, the groups with little affinity for water), the "oily" group of one molecule adhering to an "oily" group in the neighboring molecule. The rest of the groups of these molecules must, however, remain separated, since their "watery" groups (*i.e.*, the groups with strong affinity for water) cannot adhere to each other. The result is a network in which the distribution of the molecules in the water is not disturbed, since the forces of attraction between the "watery" groups of the protein molecule and the molecules of water will prevent the molecules of gelatin from attracting each other except at the one "oily" group. Since the "watery" groups prevail in bulk over the oily group of the gelatin molecule or ion, a solid network or a gel formation results instead of precipitation. Under these conditions we observe the gel form of micellæ. In a solution with much alcohol and little water (*i.e.*, on the alcohol side of the critical point) the situation becomes reversed. The forces of attraction between the "watery" groups and the solvent—which is now mainly alcohol—are weak, and since they form the bulk of the gelatin molecules the latter will attract each other in many points, thus causing a close contact over a wide area and this gives rise to the precipitation form of the micellæ. On the other hand, the few "oily" groups of the gelatin molecule may now be attracted by the alcohol and this may aid in stabilizing the solution; but at the best these forces must be weak.

In the case of crystalline egg albumin the forces of attraction between the watery group and the molecules of water are very strong and the mutual attraction of the oily groups for each other must be very feeble, since no gel formation occurs at ordinary temperature, low concentration, or a pH above 1.2. When, however, the temperature or the hydrogen ion concentration is

sufficiently raised, a change occurs—nobody knows of what nature—in the molecule, the solutions become opalescent and the albumin may set to a solid gel. In this case "oily" groups must be activated or formed which were not active or did not exist before and their natural attraction must cause the gel formation.

In the case of casein solutions in acid all the properties of the solution, stability, viscosity, osmotic pressure, and P.D., possess colloidal character. The forces of attraction between the molecules or ions of casein on the acid side of the isoelectric point for each other are very strong and for the water molecules comparatively feeble. In the case of casein chloride or casein phosphate and to a lesser extent casein nitrate, they are just strong enough to make a solution possible; but the stability of the solution depends largely on the forces set up by the Donnan equilibrium between the nascent micellæ or the existing micellæ and the surrounding solution. In the case of casein trichloracetate or casein sulphate the forces of mutual attraction between the casein molecules for each other are so much greater than those for water that these two salts are practically insoluble. On account of this great attraction for each other the granules of casein cannot swell in sulphuric acid or trichloracetic acid. Solutions of Na caseinate behave like crystalloidal solutions in regard to stability but like colloidal solutions in regard to viscosity, P.D., and osmotic pressure.

This shows the complications which may be due to the large size and complex constitution of large molecules, especially of proteins. The problems of gel formation or of precipitation are not colloidal problems, they are a part of the more general problem of solubility. These problems enter only in a secondary way into the problem of colloidal behavior, since the phenomena of aggregation are only a means of preventing the diffusion of an ion, thereby creating the conditions for the establishment of a Donnan equilibrium.

We now understand why it is not correct to define colloidal solutions as solutions in which the ultimate unit is a micella, *i.e.*, an aggregate of molecules or ions. The colloidal behavior of solutions of salts of crystalline egg albumin in regard to osmotic pressure and P.D. is only due to the non-diffusibility of the pro-

tein ion through the collodion membrane and depends in no way upon the existence of micellæ. The existence of micellæ could only diminish the value of the osmotic pressure and of the P.D. The results of our work lead to the conclusion that there is only one source of colloidal behavior, namely, the Donnan equilibrium, at least as far as the proteins are concerned. Without a Donnan equilibrium there can be no colloidal behavior of proteins. A Donnan equilibrium will always arise when the diffusion of one kind of ions is blocked while the diffusibility of oppositely charged ions is unrestricted, regardless of the nature of the block restricting the diffusibility and regardless of the nature of the ion the diffusion of which is prevented.

The writer hopes that the methods, experimental results, and theoretical conclusions described in this book may be found of use not only in the study of the colloidal behavior of other substances than proteins but also in physiology. Life phenomena cannot be dissociated from colloidal behavior, and the idea of an organism or of living matter consisting exclusively or chiefly of crystalloidal material or material with purely crystalloidal behavior is inconceivable. Organisms have been defined as chemical machines consisting essentially of colloidal material capable of growing and automatically reproducing themselves.[1] If this be true, advance in physiology will be chiefly a hit or miss game until science is in possession of a mathematical theory of the colloidal behavior of the substances of which living matter is composed. If Donnan's theory of membrane equilibria furnishes the mathematical and quantitative basis for a theory of colloidal behavior of the proteins, as the writer believes it does, it may be predicted that this theory will become one of the foundations on which modern physiology will have to rest.

[1] Loeb, J., "The Dynamics of Living Matter," New York, 1906.

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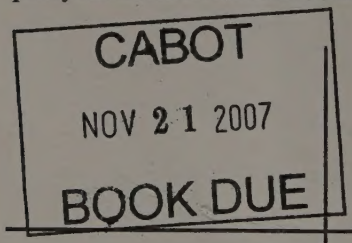

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